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REVIEW

Molecular mechanisms targeting drug-resistance and metastasis in colorectal cancer: Updates and beyond

Samar Al Bitar, Marwan El-Sabban, Samer Doughan, Wassim Abou-Kheir

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Abstract

Colorectal cancer (CRC) is the third most diagnosed malignancy and a major leading cause of cancer-related deaths worldwide. Despite advances in therapeutic regimens, the number of patients presenting with metastatic CRC (mCRC) is increasing due to resistance to therapy, conferred by a small population of cancer cells, known as cancer stem cells. Targeted therapies have been highly successful in prolonging the overall survival of patients with mCRC. Agents are being developed to target key molecules involved in drug-resistance and metastasis of CRC, and these include vascular endothelial growth factor, epidermal growth factor receptor, human epidermal growth factor receptor-2, mitogen-activated extracellular signal-regulated kinase, in addition to immune checkpoints. Currently, there are several ongoing clinical trials of newly developed targeted agents, which have shown considerable clinical efficacy and have improved the prognosis of patients who do not benefit from conventional chemotherapy. In this review, we highlight recent developments in the use of existing and novel targeted agents against drug-resistant CRC and mCRC. Furthermore, we discuss limitations and challenges associated with targeted therapy and strategies to combat intrinsic and acquired resistance to these therapies, in addition to the importance of implementing better preclinical models and the application of personalized therapy based on predictive biomarkers for treatment selection.

Key Words: Colorectal cancer; Metastatic colorectal cancer; Targeted therapy; Drug-resistance; Personalized medicine

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Core Tip: Efforts in cancer research has yielded significant advances in our understanding of the molecular mechanisms underlying colorectal cancer (CRC) resistance and metastasis. Therapeutic strategies centered on targeted molecules involved in CRC progression have been shown to be highly promising in overcoming resistance to conventional treatments. Targeted agents are currently being evaluated in preclinical and clinical studies to identify novel pharmacological targets and to study the efficacy of personalized medicine-based approaches.

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INTRODUCTION

Colorectal cancer (CRC) is among the most prevalent malignancies in the world and the third most frequent cause of cancer-related death in the US and Europe[1,2]. Estimates from the American Cancer Society indicate that over 100000 new cases of CRC will be diagnosed in 2022 in the US and 53000 deaths will result from CRC in the same year. In addition to the increased incidence of CRC, the number of patients presenting with advanced, metastatic CRC (mCRC) is increasing[3]. In fact, it has been estimated that 25% of CRC patients have mCRC at the time of diagnosis and 50% of patients subsequently develop mCRC[4].

Lifestyle factors are thought to be a major factor in the increased incidence of CRC, and they include unhealthy diet, lack of physical activity, smoking, and alcohol consumption[3]. Other factors include heredity and family history which contribute to 30% of cases and genetic mutations and variations which contribute to 10% of cases[3]. It is important for health care providers and individuals to understand the causes and risk factors of CRC, in addition to the prevention strategies that could reduce the incidence. Screening can reduce CRC incidence and death through early detection and treatment of disease[3]. Colonoscopy is the standard screening method for CRC[5]. Other imaging-based tests are also available and include computed tomography colonography, colon capsule, and flexible sigmoidoscopy. In addition, screening modalities include stool-based tests, such as fecal immunochemical testing and the multitarget stool DNA test[5].

Conventional therapy for CRC includes surgery, chemotherapy, and radiotherapy[6]. 5-fluorouracil (5-FU) is the standard treatment for mCRC. It is now being combined with other chemotherapeutic drugs to improve patient survival. 5-FU, leucovorin, and irinotecan (FOLFIRI), 5-FU, leucovorin, oxaliplatin, and irinotecan, and 5-FU, oxaliplatin, and leucovorin (FOLFOX4) have been used as multidrug chemotherapy regimens. Although these treatment strategies have improved overall survival (OS), intrinsic and acquired resistance has been a major limitation in the effectiveness of these treatments in 90% of patients with mCRC[6]. Innate resistance is usually noted during early treatment or in early clinical trials. Acquired resistance may occur through different molecular mechanisms, and is specific to each therapy; however, acquired resistance to one drug sometimes results in resistance to other drugs with the same or different mechanism of action. This is known as multidrug resistance and is responsible for multiple cross-resistance towards different drugs[7].

Chemotherapy targets rapidly dividing cells by blocking DNA replication or tubulin assembly, and thus is not specific to cancer cells and is associated with toxicity to healthy tissues[8]. In the last 15 years, major attempts have been made to develop targeted or biological therapies that kill cancer cells by targeting specific pathways implicated in tumor growth. Targeted therapies against cancer cells include mainly monoclonal antibodies (mAbs) that bind membrane growth factor receptors or their ligands, and small molecules that cross the cell membrane and inhibit cell growth and survival[9].

With the development and advancement of next generation sequencing (NGS) and omics technologies[10], it has been possible to determine molecular mechanisms underlying resistance and to develop new strategies to overcome this resistance. Over the past decade, new discoveries in the field of CRC led to the introduction of targeted therapies in clinical practice, which resulted in significant therapeutic efficacy and prolonged survival. New drugs whose action is directed at specific pathways implicated in CRC pathogenesis, including the epidermal growth factor receptor (EGFR) pathway, have been tested in preclinical models and in clinical trials. Yet, the best combination of standard chemotherapy and targeted therapy for the first-line treatment of mCRC has been debated for several years.

Understanding the mechanisms of acquired drug resistance to targeted therapies is critical for the development of novel and effective treatment combinations and will help guide future therapies. In this article, we review mechanisms of resistance to conventional therapy, we discuss the efficacy of novel targeted therapies against drug-resistant and mCRC and challenges associated with them, in addition to

strategies to overcome resistance to targeted therapy. We conclude by highlighting lessons learned from molecular studies and their clinical relevance, as well as the importance of employing novel preclinical models to facilitate the development of effective targeted therapy.

RESISTANCE TO THERAPY

Resistance to conventional treatment is one of the most challenging problems in cancer therapy, resulting in poor prognosis, recurrence, and metastasis. It is attributed to several intrinsic and acquired factors in tumor cells and in the microenvironment they reside in.

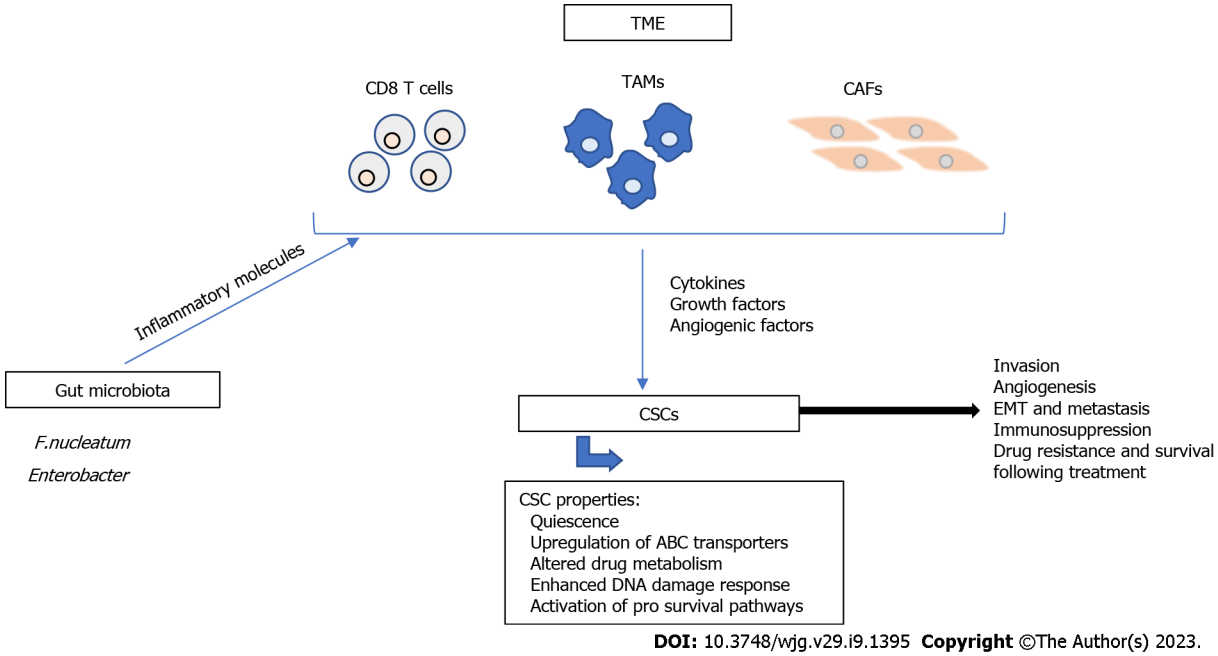
Cancer stem cells

CRC treatment requires surgical intervention, which is accompanied by the application of chemotherapy or radiotherapy, before or after surgery, as neoadjuvant or adjuvant treatment to ensure maximum reduction of tumor size[11]. These treatments are effective against cancer cells but spare the more resistant cancer stem cells (CSCs). Mechanisms of resistance are still unclear, but several factors are known to contribute to it. For example, CSCs are quiescent and do not enter the cell cycle, therefore they are not targeted by conventional therapy that kills highly proliferating cells[12]. Different molecular mechanisms are involved in CRC drug-resistance[13], as shown in Figure 1, and are summarized in this paper.

CSCs express high levels of ATP-binding cassette (ABC) transporters that mediate drug efflux and resistance to chemotherapy[14,15]. The first identified ABC member is ABCB1 or P-glycoprotein, which is expressed in normal intestinal cells. The overexpression of ABCB1 has been reported in preclinical and clinical studies of CRC and is associated with resistance to chemotherapy[16,17]. First-, second-, and third-generation inhibitors have been designed against ABCB1 and have been shown to have high affinity; however, their effectiveness is limited and needs further improvement[18]. Other ABC members include ABCC6, ABCC11, ABCF1, and ABCF2 and their upregulation has been documented in CRC tumor tissues[19], suggesting that these transporters may serve as potential targets for reversing drug-resistance in CRC.

The anti-cancer effect of chemotherapeutic drugs can be reduced by impaired drug metabolism. Capecitabine is a chemotherapeutic agent used for the treatment of mCRC. Upon administration, it is converted into 5-FU by thymidine phosphorylase (TP)[20]. It has been shown that methylation of the gene encoding TP inhibits its translation and results in resistance to capecitabine[20]. 5-FU acts by inhibiting thymidylate synthase and incorporating its metabolites into DNA and RNA[21]. Several enzymes, such as orotate phosphoribosyl-transferase and uridine monophosphate synthetase, mediate the conversion of 5-FU into its active metabolites[22]. Interestingly, lower expression of these enzymes is associated with resistance to 5-FU in CRC[23]. Additionally, TP converts 5-FU into 5-fluoro-2′ deoxyuridine and it has been shown to predict good response to 5-FU treatment and is associated with higher progression-free survival (PFS) in patients with high expression of TP[24]. Another enzyme that has been reported to affect response to chemotherapy is carboxylesterase 2. This metabolic enzyme plays a major role in the activation of irinotecan and its high expression and activity improves the efficacy of irinotecan[25]. On the other hand, uridine diphosphate glucuronosyltransferase 1A1 and β-glucuronidase inactivate irinotecan, and their alteration results in reduced irinotecan activity, suggesting that targeting these enzymes may reverse resistance to irinotecan[26,27]. Similarly, dihydropyrimidine dehydrogenase is a metabolic enzyme that mediates the catabolism of 5-FU to its inactive metabolite, and its high expression has been associated with resistance to 5-FU in CRC[28,29].

In cancers, including CRC, the DNA damage response (DDR) is activated and aberrant. This damage response consists of several kinase-dependent signaling pathways and is important for maintaining genome integrity and stability. Damage sensing is usually mediated by DDR sensors, followed by transduction of damage signals to DDR mediators and downstream molecules, leading to either cell cycle arrest, DNA damage repair, or apoptosis[30]. Ataxia telangiectasia mutated and ATM and Rad3-related protein, members of the phosphatidyl-inositol 3-kinase (PI3K) like family of protein kinases, are the main regulators of DDR. They interact with p53 and checkpoint pathways that regulate Cdc25[31]. Several mechanisms attribute to resistance of CSCs to DNA damage and include cell cycle checkpoint alteration and activation of an efficient scavenging system that protects against reactive oxygen species (ROS), which are induced by therapy[32]. Three main pathways that contribute to CRC development are unsensed or repaired by the aberrant DDR. These pathways are chromosomal instability (CIN), CpG island hypermethylation phenotype, and microsatellite instability (MSI) pathways. CIN is common in 80% of CRC cases while MSI results from inactivation of mismatch repair genes (MMR) and is common in sporadic CRC[33]. Notably, DNA repair induced by oxaliplatin is mainly mediated by the nucleotide excision repair pathway[34]. The upregulation of excision repair cross-complementing 1 has been linked to oxaliplatin resistance in CRC[34] and its mRNA expression level is a predictive marker of survival in patients treated with 5-FU and oxaliplatin[35]. These results suggest that the expression levels of DNA repair proteins may serve as treatment response biomarkers, and the reduction of their expression can enhance the effect of DNA-damaging agents, leading to eradication of resistant CSCs.



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Figure 1 Major mechanisms of cancer stem cell resistance. Cancer stem cell (CSC) resistance has been associated with CSC characteristics including quiescence, upregulation of ATP-binding cassette transporters, altered drug metabolism, enhanced DNA damage response, and activation of pro survival pathways. The tumor microenvironment (TME) plays a major role in the resistance of CSCs to therapy. CD8 T cells, tumor associated macrophages, and cancer associated fibroblasts (CAFs) are major components of the TME and contribute to tumor progression and metastasis through the secretion of cytokines, growth factors, and angiogenic factors. Additionally, gut microbiota, such as *Fusobacterium nucleatum* and *Enterobacter* secrete inflammatory molecules that modulate the TME and contribute to therapy resistance. All of these mechanisms contribute to tumor invasion, angiogenesis, epithelial-to-mesenchymal transition, immunosuppression, drug resistance and survival following treatment. TAMs: Tumor associated macrophages; CAFs: Cancer associated fibroblasts; EMT: Epithelial-to-mesenchymal transition.

Intrinsic and acquired resistance to apoptosis is one of the characteristics of CSCs. Apoptosis is regulated by a balance between pro-apoptotic, anti-apoptotic, and pro-survival mechanisms[36], which is frequently altered in cancer, including CRC[34,37]. p53 plays a key role in the induction of apoptosis in response to DNA damage by chemotherapy[34]. However, p53 is mutated in 85% of CRC cases and is linked to resistance to 5-FU and oxaliplatin[38]. In addition, the expression of high levels of anti-apoptotic proteins, including Bcl-2 family members, is a characteristic of CSCs and results in resistance to cell death by apoptosis[39]. Frameshift mutations in the *BAX* gene result in the loss of expression and activity of the anti-apoptotic protein BAX, leading to chemoresistance[34]. Other anti-apoptotic proteins that are implicated in chemoresistance include Bcl-XL and the FLICE-inhibitory protein[40].

Moreover, several pro survival signaling pathways are activated in CRC. One major pathway is the Wnt/β-catenin pathway, which is important for stemness and resistance. Binding of Wnt ligand to the Frizzled receptor results in activation of β catenin, a key effector in this pathway[41]. Activation of the Wnt pathway induces proliferation and differentiation of CSCs, which is partly mediated by activation of several molecules that are recognized as putative CSC markers and include Lgr5, CD44, CD133, and Epcam[42]. All of these markers are associated with CSC resistance to chemo- and radiotherapy. Other pathways that are involved in stemness include the Notch and Hedgehog pathways[42].

Tumor microenvironment

CRC resistance has been also linked to the tumor microenvironment (TME) that is also involved in the multistep process that encompasses the development of adenomatous polyps from normal colonic epithelium, finally leading to invasive CRC[43,44]. The TME consists mainly of immune cells, endothelial cells, stromal cells, extracellular matrix (ECM), and signaling molecules[45]. Solid tumors, including CRC are infiltrated by different cells, such as dendritic cells, monocytes, neutrophils, CD8 and CD4 T cells, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and mesenchymal stem cells. During tumor formation, interactions between tumor and stromal cells and secretion of soluble inflammatory molecules mediate the attraction of immune cells that promote tumor cell survival and metastasis[45,46]. The most important tumor-promoting cells are TAMs and CAFs. These cells facilitate tumor progression through direct contact with other cells or through secretion of cytokines, growth factors, and angiogenic factors, thereby promoting ECM formation, tumor invasion, angiogenesis, epithelial-to-mesenchymal transition (EMT), and immunosuppression[43,45].

Gut microbiota

Strong evidence is emerging to support the role of gut microbiota in the progression and resistance of

CRC and interventions made in this regard may hold promises for improving CRC treatment[47]. *Fusobacterium nucleatum* has been shown to contribute to CRC chemoresistance through activation of innate immune signals that stimulate the autophagy pathway[48]. The use of antibiotics can increase pathogenic bacteria such as *Enterobacter* and has been shown to reduce the anti-cancer effect of oxaliplatin through modulation of cytokine secretion and ROS production in the TME[49]. On the other hand, the effect of immunotherapy has been shown to be enhanced by intestinal microbiota, such as *Faecalibacterium*, *Clostridiales*, and *Bifidobacterium spp*[50,51]. The exact mechanism of action is still unclear but has been attributed to direct interactions between these bacteria and immune cells[52], in addition to a possible role for microbial metabolites, such as butyrate and propionate[53].

TARGETED THERAPY

Targeted agents can directly inhibit the proliferation and migration of cancer cells (Figure 2). They could also target the TME, thereby limiting tumor growth and enhancing immune surveillance. Small molecules play a major role in such treatments, as they can penetrate cells to selectively inactivate specific enzymes involved in tumor proliferation induction and apoptosis inhibition[54].

Targeting EGFR

EGFR belongs to the ErbB family of receptor tyrosine kinases and is involved in cellular proliferation, survival, migration, adhesion, and angiogenesis[55,56]. 80% of CRCs express or upregulate the *EGFR* gene[57,58], and this expression is associated with a risk of metastasis[59], therefore inhibiting EGFR could be a possible strategy to reduce cellular proliferation.

EGFR activation can be blocked by mAbs or tyrosine kinase inhibitors (TKIs). EGFR mAbs include cetuximab and panitumumab, which are currently used in parallel with FOLFOX or FOLFIRI regimens in the treatment of patients with *KRAS* or *NRAS* wild-type (WT) tumors[60]. In RAS-mutant tumors, constitutive activation of signaling pathways downstream of EGFR limits the effectiveness of EGFR inhibitors[61].

Cetuximab is a chimeric murine human IgG1 mAb that binds to the extracellular domain of EGFR and inhibits its pro-oncogenic action in cancer cells[62,63] (Table 1 and Figure 2). It also binds to natural killer cells and induces antibody-dependent cell-mediated cytotoxicity[62]. In a study that involved patients with advanced CRC after treatment with irinotecan, treatment with cetuximab alone or in combination with irinotecan showed significant clinical activity, with an enhanced rate of response and median survival time in the combination groups[64]. Combining cetuximab with FOLFIRI reduced the risk of progression of mCRC by 15% in first-line treatment of patients with *KRAS* WT tumors, when compared to FOLFIRI alone[65]. Complete or partial tumor responses were observed in 46.9% of patients treated with combination therapy and in 38.7% of patients treated with FOLFIRI alone[65]. Another treatment regimen that was tested in the first-line treatment of mCRC included FOLFOX4 and cetuximab[66]. Results from this randomized study showed an increased chance of response and lower risk of disease progression in the combination-treated group when compared to FOLFOX4 alone in *KRAS* WT patients[66]. A more recent randomized phase 3 Medical Research Council COIN trial showed that adding cetuximab to oxaliplatin-based chemotherapy increased the response rate in patients with advanced CRC; yet no enhancement of PFS or OS was shown[67].

Similar to cetuximab, treatment with panitumumab alone or in combination with standard chemotherapy has shown promising results in several clinical trials[60,68]. Panitumumab monotherapy was effective in CRC patients with *KRAS* WT tumors, with a response rate of 17%[69]. In an open-label phase III trial that involved patients with chemotherapy-refractory mCRC, panitumumab plus best supportive care (BSC) significantly prolonged PFS when compared to BSC alone. Response rates were 10% for panitumumab and 0% for BSC, with no difference observed in OS[70]. Several clinical trials were conducted to compare the efficacy of panitumumab and FOLFOX4 in comparison to FOLFOX4 alone[60,68]. Results from a phase III trial showed that combination treatment significantly improved PFS whereas the increase in OS was insignificant when compared to FOLFOX4 alone in *KRAS* WT tumors[60]. Except for the toxicities that are usually associated with EGFR inhibitors, adverse event rates were comparable between these treatments[60]. The very recent PARLIM trial showed that PFS and OS were improved upon the addition of panitumumab to FOLFOX in *KRAS* WT CRC patients with R0/1-resected liver metastases. Importantly, no new adverse events were observed in the combination-treated group[71].

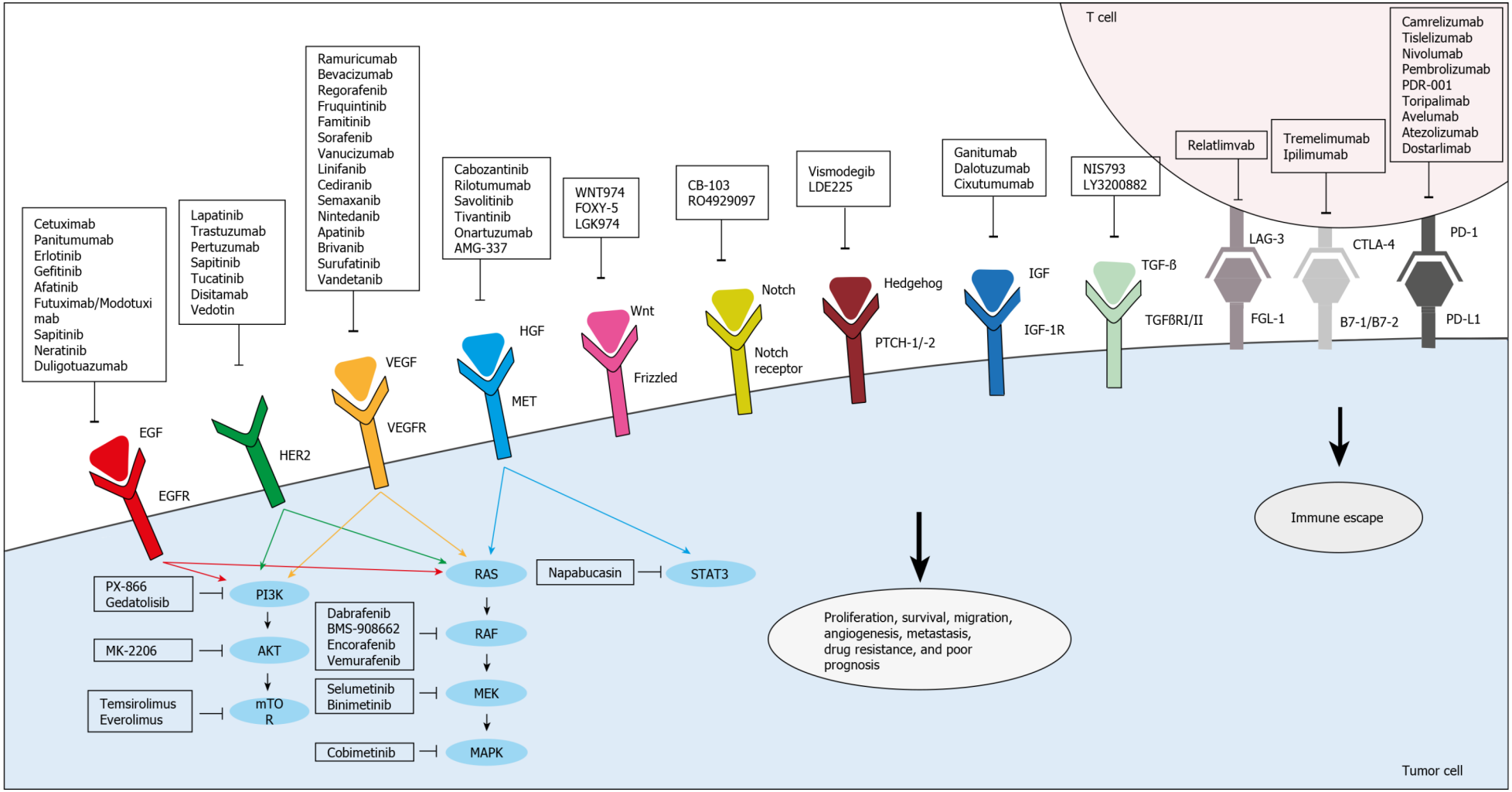
The most common side effects observed in trials of these EGFR mAbs were skin toxicity, abdominal pain, nausea, diarrhea, infusion reactions, fatigue, and hypomagnesemia. Rare adverse events included pulmonary fibrosis, sepsis, severe skin toxicity, and anaphylaxis[72].

EGFR TKIs are small molecules derived from quinazolines that block the tyrosine kinase domain of different receptors, including EGFR. Erlotinib is specific to EGFR alone and is used to block ligand-induced EGFR receptor phosphorylation[73]. Gefitinib is another EGFR TKI that has a similar mechanism of action to erlotinib, but also targets other pathways, such as the extracellular signal-related kinases 1/2 (ERK1/2) pathway in mesothelioma cell lines[73].

Table 1 Agents targeting epidermal growth factor receptors and downstream molecules under clinical investigation for the treatment of drug-resistant and metastatic colorectal cancer

Agent	Targeted molecule	Condition	Study phase	Clinical trial identifier
Erlotinib	EGFR	First-line treatment for mCRC	Phase III	NCT01229813
Futuximab/Modotuximab (Sym-004)	EGFR	mCRC	Phase II	NCT02083653
Gefitinib	EGFR	Refractory CRC	Phase I/II	NCT00242788
Afatinib	EGFR	Refractory mCRC	Phase II	NCT01919879
		Advanced CRC	Phase II	NCT00801294
		mCRC	Phase II	NCT01152437
Dabrafenib (GSK2118436)	BRAF	mCRC	Phase II	NCT03668431
		mCRC	Phase II	NCT03428126
BMS-908662	BRAF	K-RAS/BRAF-mutated CRC	Phase I/II	NCT01086267
Encorafenib	Wild-type and BRAF V600E	Previously untreated BRAF-mutant mCRC	Phase II	NCT03693170
Vemurafenib	Mutated BRAF V600E	BRAF V600E mutated advanced CRC	Phase II	NCT03727763
PX-866	PI3K	mCRC	Phase I/II	NCT01252628
Gedatolisib	PI3K/mTOR	KRAS/NRAS-wild-type mCRC	Phase II	NCT01925274
		mCRC	Phase I/II	NCT01937715
Temsirolimus CCI-770	mTOR	KRAS-mutated mCRC	Phase II	NCT00827684
		Cetuximab-refractory CRC	Phase I	NCT00593060
Everolimus (RAD001)	mTOR	mCRC	Phase II	NCT01387880
		mCRC	Phase I/II	NCT01058655
		Advanced mCRC	Phase I/II	NCT01139138
		Refractory mCRC	Phase I	NCT01154335
MK-2206	AKT	Advanced CRC	Phase II	NCT01333475
Napabucasin (BBI608)	STAT3	Previously treated mCRC	Phase III	NCT03522649
Cobimetinib	MAPK	mCRC	Phase III	NCT02788279
Selumetinib	MEK	mCRC	Phase II	NCT00514761
Binimetinib	MEK	Previously untreated BRAF-mutant mCRC	Phase II	NCT03693170
Neratinib	EGFR/HER2/4	KRAS/NRAS/BRAF/PIK3CA-wild-type mCRC	Phase II	NCT03457896
Sapitinib (AZD-8931)	EGFR/HER2/3	mCRC	Phase II	NCT01862003
Duligotuzumab (MEHD7945A)	EGFR/HER3	KRAS-mutated mCRC	Phase II	NCT01652482
Trastuzumab	HER2	First-line HER2-positive mCRC	Phase III	NCT05253651
Tucatinib	HER2	First-line HER2-positive mCRC	Phase III	NCT05253651
Disitamab Vedotin	HER2	HER2-positive advanced CRC	Phase II	NCT05493683
		HER2-expressing mCRC	Phase II	NCT05333809
Trastuzumab-emtansine	HER2	HER2-positive mCRC progressing after trastuzumab and lapatinib	Phase II	NCT03418558

EGFR: Epidermal growth factor receptor; mCRC: Metastatic colorectal cancer; PI3K: Phosphoinositide 3-kinases; mTOR: Mammalian target of rapamycin; STAT3: Signal transducer and activator of transcription 3; AKT: Protein kinase B; MAPK: Mitogen-activated protein kinases; MEK: Mitogen-activated extracellular signal-regulated kinase; HER2: Human epidermal growth factor receptor 2.



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Figure 2 Targeted therapies under investigation for the treatment of drug-resistant and metastatic colorectal cancer. Anti-epidermal growth factor receptor, anti-vascular endothelial growth factor/vascular endothelial growth factor receptor, and anti-human epidermal growth factor receptor 2 agents inhibit their respective targets and thus, the downstream effector pathways, PI3K/Akt and RAS/RAF. Other agents directly target and inhibit PI3K, AKT, mammalian target of rapamycin, RAF, mitogen-activated extracellular signal-regulated kinase, or mitogen-activated protein kinases. In addition, anti-hepatocyte growth factor/mesenchymal epithelial transition factor receptor agents target this pathway to inhibit signal transducer and activator of transcription, which is also targeted by Napabucasin. Several novel agents that are aimed at other pathways implicated in colorectal cancer proliferation, survival, resistance, and metastasis are also being evaluated. Targeted pathways include Wnt, Notch, Hedgehog, insulin growth factor/insulin growth factor receptor-1, and transforming growth factor beta. Moreover, immune escape can be hindered through immunotherapy which targets co-inhibitory molecules, mainly programmed

death-1/programmed death ligand-1, cytotoxic T lymphocyte-associated antigen 4, and lymphocyte activation gene 3. EFGR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; HER2: Human epidermal growth factor receptor 2; mTOR: Mammalian target of rapamycin; MEK: Mitogen-activated extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinases; HGF: Hepatocyte growth factor; MET: Mesenchymal epithelial transition factor receptor; STAT3: Signal transducer and activator of transcription; CRC: Colorectal cancer; IGF: Insulin growth factor; IGF-1R: Insulin growth factor receptor-1; TGF-β: Transforming growth factor beta; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; LAG-3: Lymphocyte activation gene 3; EGF: Epidermal growth factor; TGFβRI/II: Transforming growth factor-Beta type I/II.

It is important to note that studies investigating the efficacy of EGFR targeted therapy vary widely in clinical context, and some focus on the effect of EGFR monotherapy while others compare it to a combination of various chemotherapy regimens. One important factor to be taken into consideration is *KRAS* status, which could be used as a biomarker to predict the effectiveness of a treatment. Several inhibitors targeting EGFR or downstream molecules are currently under clinical investigation and are summarized in Table 1.

Targeting HER

Human EGFR 2 (HER2) is emerging as a key driver in CRC. It acts similar to EGFR, as they both share common downstream pathways, such as RAS/RAF/MEK and PI3K/AKT, which explains the link between HER2 overexpression and resistance to EGFR inhibitors[74,75]. The *HER2/neu* oncogene encodes a receptor with intrinsic tyrosine kinase activity[76]. HER2 lacks an endogenous ligand unlike other members of the HER/EGFR/ERBB system[77]. Homodimerization or heterodimerization with other EGFR family receptors, HER3 and EGFR, results in transphosphorylation of tyrosine residues within the cytoplasmic domain of HER2, thus leading to its activation[77,78]. HER2-HER3 heterodimers activate the PI3K/AKT pathway which is implicated in cancer cell growth and survival[79].

Different rates of HER2 amplification have been reported in CRC[80-82], with rates of membranous expression ranging from 2.1% to 11%[80,83,84], and that of cytoplasmic expression ranging from 47.4 to 68.5%[80,85,86]. Several factors may account for this variability, including small sample size, different antibodies used for immunohistochemistry (IHC), and analysis of different subgroups of patients with multiple clinical characteristics[87]. The efficacy of targeted agents against HER2-expressing CRC was determined in several clinical trials. Ramanathan *et al*[88] reported the detection of HER2/neu overexpression in only 8% of screened tumors in patients with advanced CRC and this low overexpression rate limited the study of irinotecan and trastuzumab, a humanized mAb targeting the HER2/neu receptor, in a phase II clinical study. Yet, partial response was observed in some patients, and the response was maintained for approximately six wk[88]. In a proof-of-concept study that exploited patient-derived xenografts (PDX), HER2 was identified as an effective therapeutic target in cetuximab-resistant mCRC [89]. HER2 amplification was detected in clinically unresponsive *KRAS* WT patients, and the combination of lapatinib (a dual EGFR/HER2 TKI) and pertuzumab induced an increase in response rate and tumor regression, in agreement with clinical studies in patients with similar clinicopathological characteristics[89]. The synergic antiproliferative effect of HER2 and EGFR blockade was also demonstrated in cetuximab-resistant CRC cell lines[74,90]. Interestingly, HER2 activating mutations were identified in CRC PDX and were shown to be highly sensitive to HER2/EGFR TKIs neratinib and afatinib and resulted in tumor regression when subjected to dual HER2 targeted therapy with trastuzumab plus TKIs[91]. It was also reported that these mutations cause oncogenic transformation of colon epithelial cells and resistance to anti-EGFR monotherapy[91]. Various clinical trials targeting

HER2 alterations in combination with chemotherapeutic therapies in patients with mCRC have validated findings from preclinical studies. High toxicity[92] and poor accrual[88,93] were the reasons behind halting earlier clinical studies evaluating the addition of HER2 mAbs (trastuzumab or pertuzumab) to cetuximab or chemotherapy (*i.e.*, irinotecan, 5-FU, and oxaliplatin). In a phase I trial involving patients with HER2-positive refractory tumors, none of the CRC patients responded to the combination of trastuzumab, paclitaxel, and interleukin (IL)-12[94]. More recently, a study that followed the stringent HERACLES criteria reported that the combination of trastuzumab and lapatinib achieved an objective response rate of 30% and was well tolerated in *KRAS* codon 12/13 WT, HER2-positive mCRC patients[95]. Within the same project, HERACLES-B phase II trial assessed the efficacy of pertuzumab and trastuzumab emtansine; however, it did not reach its primary endpoint of response rate. Yet, this combination can be considered a potential therapeutic strategy for HER2-positive mCRC, based on the high disease control achieved, in addition to the enhanced PFS and low toxicity[96]. The MyPathway trial assessed the combination of pertuzumab and trastuzumab in pretreated HER2-amplified mCRC patients and further supported the efficacy of the dual blockage of HER2[97,98]. Several agents targeting HER and EGFR are currently under clinical investigation (Table 1).

Targeting VEGF

Angiogenesis is the formation of new blood vessels from endothelial cells. It is mediated by vascular endothelial growth factor (VEGF), together with platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)[99]. Angiogenesis plays an important role in tumor initiation, growth, and metastasis. The VEGF system consists of six ligands and three receptors known as VEGF receptors (VEGFR). VEGF-A is secreted by multiple cell types, including cancer cells, and plays a major role in survival, growth, differentiation, and migration of endothelial cells[100]. VEGF-A mediates its effect by binding to VEGFR2, which is the major signal transducer of angiogenesis and is expressed by endothelial cells. On the other hand, VEGFR1 is a strong VEGF inhibitor[101]. Hypoxia is a key regulator of angiogenesis in cancer through hypoxia-inducible factors, which induce transcription of several genes, including VEGF-A[102].

VEGF levels and VEGFR activity are elevated in patients with CRC and are associated with poor prognosis[103]. The activation of this system is important both in local sites to support tumor progression and in metastatic sites to support neovascularization and tumor survival; therefore, a targeted therapy against VEGF/VEGFR might be developed at all stages of tumor progression and metastasis. Like EGFR, targeted therapy against angiogenesis consists of mAbs and TKIs. mAbs bind to VEGF-A or block the extracellular domain of its receptor. mAbs that bind VEGF-A include bevacizumab and aflibercept, thereby preventing activation of their receptors. Ramucirumab binds to the VEGFR2 extracellular domain, inhibiting the binding of VEGF ligands, thereby inhibiting receptor activation [104].

Bevacizumab as a monotherapy has a limited effect and is therefore used in combination with chemotherapy in first- and later-lines of mCRC treatment[105]. It is the first Food And Drug Administration (FDA)-approved VEGF-targeted agent for mCRC[105]. The first randomized clinical trial showed that bevacizumab improves response rate, PFS, and OS, thereby enhancing chemotherapy efficacy[106]. Combining bevacizumab (5 mg per kg of body weight every two wk) with irinotecan, 5-FU, and leucovorin (IFL) enhanced median duration of survival and PFS, as compared to IFL treatment alone, corresponding to a hazard ratio for death of 0.66 and for disease progression of 0.54, respectively[106]. The results also showed that median duration of the response to combination treatment was 10.4 mo as compared to 7.1 mo in the group treated with IFL and placebo[106]. A major adverse event was grade 3 hypertension which was more common in the group treated with IFL and bevacizumab but was easily managed. More recent trials showed that modern combination regimens were better substitutes for IFL; however, the efficacy of combining bevacizumab with first-line treatment of mCRC has been controversial. Several recent clinical trials demonstrated the promising efficiency of combining bevacizumab with trifluridine/tipiracil, which is usually better tolerated than capecitabine, especially in elderly patients with mCRC[107-109]. Notably, promising results were reported in the phase II TASCO study that assessed the effectiveness of combining bevacizumab with trifluridine/tipiracil as first-line treatment in untreated patients with unresectable mCRC[110]. This combination treatment achieved better median PFS and OS when compared to patients receiving bevacizumab plus capecitabine. On the other hand, Chen *et al*[111] carried out a meta-analysis that showed no improvement in OS upon the addition of bevacizumab to FOLFOX/FOLFIRI/capecitabine plus oxaliplatin (XELOX) regimens when compared to chemotherapy alone, unless PFS is considered, specifically in capecitabine-based regimens. This exception was established based on two trials, the NO16966 study[112] and ITACA trial[113], which used PFS as an endpoint measurement. These studies showed that adding bevacizumab to oxaliplatin-based therapy (XELOX or FOLFOX4) significantly improved PFS in patients with mCRC [112]. OS and response rate were not changed by the addition of bevacizumab, suggesting that prolonged treatment may be needed for optimal combination efficacy[112]. Interestingly, it has been documented that both patients with *KRAS* mutations and with WT *KRAS* may benefit from adding bevacizumab to chemotherapy[114,115]. The efficacy of the second-line application of bevacizumab has also been validated in several trials that showed longer PFS and OS, and a better response rate, compared with standard chemotherapy alone in the E3200 study[116] and III ML18147 trial[117].

The addition of aflibercept to FOLFIRI enhanced the survival of patients progressing who were previously given oxaliplatin-based regimens[118]. Combination treatment resulted in a 9% increase in response rate, accompanied by an improvement in PFS from 4.7 to 6.9 mo and OS from 12.1 to 13.5 mo [118].

Ramucirumab was approved by the FDA for second-line treatment of mCRC based on the phase III RAISE trial[119]. Data from this study showed that the addition of ramucirumab significantly prolonged PFS and OS but not response rate, following first-line treatment with 5-FU, oxaliplatin, and bevacizumab[119].

Few VEGF TKIs have been proven to be effective in patients with mCRC. These include regorafenib, which was approved by FDA for the treatment of mCRC[120]. Yet, regorafenib has multiple targets, other than VEGF, whereby it also inhibits PDGF receptor, FGF receptor, and BRAF[120]. Notably, treatment of mCRC patients with regorafenib was associated with enhanced OS[121]. A more significant OS benefit was observed when combining regorafenib with its major metabolites, M-2 and M-5, in concentrations ranging between 2.5 and 5.5 mg/L[121]. While no improvement in the response rate was shown upon adding regorafenib to FOLFOX in mCRC patients as compared to chemotherapy alone [120], better median OS and PFS were achieved using regorafenib alone than placebo for refractory mCRC treatment in the phase III CORRECT trial[122]. These results were also validated in an Asian population in the CONCUR trial[123]. Anlotinib, a novel TKI that inhibits VEGFR1/2/3, among other kinases, showed an enhanced overall rate response and PFS when combined with capecitabine and oxaliplatin in the first-line treatment of mCRC[124]. Other TKI agents have been developed in the last few years, these include fruquintinib[125] and famitinib[126], in addition to other agents that are under clinical investigation and are summarized in Table 2.

Targeting MEK and mutant BRAF

BRAF mutations are found in 8% to 12% of mCRC cases, and the V600E-activating mutations, which are the most prevalent mutations, are most commonly located in right-colon tumors, and confer a worse prognosis for mCRC[127,128]. *BRAF* mutations are generally mutually exclusive with *KRAS* and *NRAS* mutations. Notably, BRAF and RAS are the only available biomarkers for advanced CRC that are used in clinical practice[129].

BRAF is a downstream effector of RAS in the EGFR pathway and several preclinical studies have shown that BRAF inhibition may induce EGFR overactivation and that EGFR inhibition is important for sensitizing resistant cell lines to anti-BRAF agents[130]. In fact, BRAF inhibitor monotherapy in CRCs harboring V600E-activating mutations is ineffective with a response rate of only 5%[131]. Capalbo *et al* [132] reported the first clinical evidence that combining anti-EGFR (panitumumab) and an inhibitor of BRAF V600 kinase (vemurafenib) achieves strong disease control and is well tolerated in patients with mCRC that progressed on standard lines of treatment. However, this is only achieved in *RAS* and *BRAF* WT tumors, as *RAS* and *BRAF* mutations lead to the constitutive activation of downstream transducers of EGFR, circumventing EGFR inhibition, resulting in failure of anti-EGFR therapy[133-135]. A very recent randomized trial reported that the addition of vemurafenib to irinotecan combined with cetuximab improved PFS (hazard ratio of 0.50) in patients with *BRAF*-mutated, *RAS* WT mCRC. The response rate was 17% upon addition of vemurafenib and 4% without vemurafenib[136]. Disease control rate was also improved by 44%, suggesting that blocking signaling activity of EGFR using cetuximab prevents its feedback upregulation by vemurafenib. Interestingly, treatment with EGFR and BRAF inhibitors led to a decline in circulating tumor DNA (ctDNA) BRAF V600E variant allele frequency in 87% of the studied population[136]. In the phase III BEACON CRC trial, twenty-nine patients with *BRAF V600E*-mutant mCRC who had experienced treatment failure with chemotherapy were selected to assess the safety of the encorafenib, binimetinib, and cetuximab regimen. The results showed that the tolerability of this treatment regimen was acceptable, with an overall response rate of 48%, median PFS of 8.0 mo, and median OS of 15.3 mo[137].

BRAF V600E mutations result in constitutive activation of BRAF kinase, which results in activation of mitogen-activated protein kinase (MAPK) kinases MEK1 and MEK2. The latter phosphorylates and activates ERK kinases, resulting in phosphorylation and activation of key molecules involved in proliferation and survival[138].

Studies have shown that combination therapies targeting RAF and EGFR or RAF and MEK can inhibit feedback reactivation of the MAPK signaling pathway, resulting in more robust inhibition and improved efficacy of the treatment in *BRAF*-mutant CRC[139,140]. Combining RAF and MEK inhibitors produced a 12% partial response and 2% complete response, with a more than 36 mo duration of response, whereby 56% of the patients achieved stable disease. Interestingly, 9 patients who remained in the study for more than 6 mo had reduced levels of phosphorylated ERK during treatment, relative to pretreatment biopsies[141]. A clinical trial of combined inhibition of BRAF, EGFR, and MEK with dabrafenib, panitumumab, and trametinib, respectively, showed improved efficacy in patients with *BRAF V600E*-mutant CRC[140]. Interestingly, the triplet regimen achieved a response rate of 21% that was higher than dabrafenib and panitumumab (10%) or panitumumab and trametinib (0%)[140]. The BEACON trial reported similar results, whereby a triple treatment consisting of cetuximab, encorafenib, and binimetinib (a MEK inhibitor) significantly prolonged OS and achieved a higher response rate than standard chemotherapy, with a comparable rate of adverse events[142]. Few agents targeting mutant

Table 2 Agents targeting vascular endothelial growth factor/vascular endothelial growth factor receptor under clinical investigation for the treatment of drug-resistant and metastatic colorectal cancer

Agent	Targeted molecule	Condition	Study phase	Clinical trial identifier
Vanucizumab	VEGF-A/angiopoietin-2	mCRC	Phase II	NCT02141295
Sorafenib	VEGFR	mCRC	Phase II	NCT03251612
		Previously treated mCRC	Phase II	NCT01471353
		mCRC	Phase II	NCT00826540
		KRAS-mutated mCRC	Phase II	NCT01715441
Bevacizumab	VEGF	Untreated mCRC	Phase II	NCT02141295
		Advanced CRC	Phase II	NCT02487992
Linifanib ABT-869	VEGFR	Advanced CRC	Phase II	NCT00707889
Vatalanib	VEGFR	mCRC	Phase III	NCT00056446
		mCRC	Phase III	NCT00056459
Famitinib	VEGFR2/3	Advanced CRC	Phase II	NCT01762293
Cediranib	VEGFR2	First-line mCRC	Phase III	NCT00399035
Semaxanib	VEGFR	mCRC	Phase III	NCT00004252
		Advanced CRC	Phase I/II	NCT00005818
Nintedanib	VEGFR	Refractory mCRC	Phase III	NCT02149108
Ramucirumab	VEGFR2	Chemotherapy refractory mCRC	Phase III	NCT03520946
Apatinib	VEGFR2	Refractory CRC	Phase II	NCT03190616
		mCRC	NA	NCT03743428
		End-stage CRC	Phase II	NCT02829385
Brivanib	VEGFR2	KRAS-wild-type mCRC	Phase III	NCT00640471
Regorafenib	VEGFR1/2/3	Later-lines treatment of mCRC	Phase III	NCT05328908
		mCRC	Phase III	NCT05425940
Surufatinib	VEGFR1/2/3	Advanced CRC	Phase II	NCT05372198
Lenvatinib	VEGFR1/2/3	mCRC	Phase III	NCT04776148
Fruquitinib	VEGFR tyrosine kinase	Non-MSI-H/dMMR mCRC	Phase II	NCT04866862
Vandetanib	VEGF/VEGFR	mCRC	Phase I	NCT00532090
		mCRC	Phase II	NCT00500292
		Advanced CRC	Phase I	NCT00496509

VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; mCRC: Metastatic colorectal cancer; MSI: Microsatellite instability; dMMR: mismatch repair deficient.

BRAF or *MEK* have been tested in clinical settings in the context of mCRC (Table 1).

The most common adverse events associated with BRAF inhibition include rash, fatigue, arthralgia, and diarrhea. When combined with MEK inhibitors, toxicities include pulmonary toxicities and ophthalmic changes[143].

Targeting c-MET and HGF

MET is activated by hepatocyte growth factor (HGF) that is secreted by cells of mesenchymal lineage [144]. The MET pathway is frequently aberrantly activated in CRC, in which its overexpression has been reported in up to 70% of cases[144]. MET has been proposed to be a major contributor to resistance to anti-angiogenic therapy and is associated with progression, metastasis, and poor prognosis[145,146], due to c-MET activation of several proteins, such as surviving and x-linked inhibitor of apoptosis protein[147]. In fact, inhibition of the VEGF pathway results in upregulation of MET. A study reported that resistance to cetuximab was caused by MET locus amplification in CRC PDX and that treatment with a MET inhibitor led to an anti-tumor effect[148].

Various mAbs and small molecules with different mechanisms of action have been developed to target the HGF-MET pathway in mCRC[9]. Some drugs are directed at blocking HGF activation and production, while other drugs inhibit the binding of HGF to MET receptors. Agents that interfere with the binding of HGF to MET can be classified as MET antagonists, which competitively bind to MET receptors or as MET TKIs, which inhibit intracellular tyrosine kinase activity[9].

Cabozantinib is a multi-kinase inhibitor that targets MET and VEGFR2, in addition to other kinases [146]. A study reported a potent growth inhibitory effect of cabozantinib in 80% of tumors treated using a CRC PDX model and this inhibition was mostly observed in tumors with *PIK3CA* mutation. Mechanistically, cabozantinib inhibited Akt activation and decreased the expression of genes involved in the PI3K pathway[146]. Several clinical trials assessed the efficacy of agents that neutralize HGF and block its ability to bind to the MET receptor. A randomized phase Ib/II trial of panitumumab in combination with rilotumumab (a human mAb against HGF), ganitumab (a human mAb against insulin-like growth factor 1 receptor), or placebo in patients with *KRAS* WT mCRC showed a significant increase in overall response rate of 10% when combining panitumumab with rilotumumab[149]. However, the enhancement in response rate did not translate into significant improvement in OS and PFS. Agents, such as onartuzumab, that compete with HGF for binding to MET have been developed and tested in various solid tumors, including CRC. A phase II randomized trial of first-line FOLFOX plus bevacizumab with or without onartuzumab (MET inhibitor) reported an improvement in PFS in the MET IHC-negative population with mCRC, as compared to those receiving treatment without onartuzumab[150]. However, the addition of onartuzumab did not improve OS or response rate in this population[150]. Tivantinib is an oral small molecule allosteric receptor TKI that selectively keeps MET in the inactive state[151]. In the case of mCRC, clinical trials of tivantinib are insufficient to evaluate its efficacy. A phase I/II trial involving CRC patients with WT *KRAS* receiving tivantinib or placebo plus cetuximab and irinotecan found no PFS improvement[152]. A recent phase II trial of tivantinib and cetuximab in patients with MET-high *KRAS* WT mCRC did not meet its primary endpoint; yet, results suggested some efficacy of the combination, with approximately 10% of patients achieving an objective response[153]. Merestinib, an oral multikinase inhibitor, demonstrated an acceptable safety profile and potential anti-tumor effect in a recent first-in-human phase I study involving patients with advanced cancer, including CRC[154]. Findings from this study warrant further investigation to determine the efficacy of this agent in patients with *KRAS* WT mCRC.

Mild adverse events have been reported for the above-mentioned agents, including fatigue, poor appetite, allergic reactions, edema, skin rash, and neutropenia[155,156].

AMG-337, an oral ATP-competitive TKI specific to MET, is being investigated in a CRC phase I trial (Table 3). Crizotinib targets TKIs of MET, in addition to macrophage-stimulating 1 receptor and ROS proto-oncogene[157]. Although there is a lack of clinical evidence for crizotinib in CRC, a series of trials are in progress[158] (Table 3). The use of crizotinib might enhance the response to radiation therapy in *KRAS*-mutant CRC cell lines, and a combination of crizotinib with mitomycin C seemed to have a synergistic effect against CRC in preclinical results, which showed promise for future anti-CRC treatments[159]. Few MET inhibitors are under clinical investigation for the treatment of mCRC, and several new agents are being tested in patients with CRC (Table 3).

Immune checkpoint inhibitors

In addition to developing agents to directly target pathways involved in tumor growth and metastasis, there is great interest in modulating other pathways involved in immune recognition and responses against cancer cells (Table 4). Immune escape has been frequently identified in various cancers, including CRC[160]. Underlying mechanisms include secretion of immunosuppressive cytokines (transforming growth factor beta (TGFβ), IL-6, CXCL3, CXCL4, and high mobility group box-1), recruitment of regulatory T cells, and loss of immunogenicity *via* downregulation of major histocompatibility complex-I (MHC-I)[161,162]. Tumor activation of co-inhibitory receptors, also known as immune checkpoint receptors, on the surface of T cells results in T cell inactivation and exhaustion[163]. These receptors include programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4)[164]. PD-1 is expressed on peritumoral lymphocytes and is activated by its ligands [programmed death ligand-1 (PD-L1) or PD-L2], which are expressed on tumor cells, to suppress immune functions[165]. mCRC lesions express higher levels of PD-L1 than primary lesions[166], paving the way for promising clinical benefits. Six antibodies against PD-1 or PD-L1 have been approved by the FDA as an anti-cancer treatment, among which some have been evaluated in mCRC patients[167,168]. Metastatic DNA mismatch repair-deficient (dMMR)/MSI-high (MSI-H) CRC has a poor prognosis and is less responsive to conventional chemotherapy, which could be linked to *BRAF* mutation[169,170]. Importantly, patients who have high mutational tumor burden, with dMMR or MSI-H, respond to immune checkpoint targeted therapy[171-173], most probably due to the fact that mutations result in tumor neoantigens that attract T cell infiltration[174]. Pembrolizumab was the first PD-1 inhibitor to be approved by the FDA for the treatment of mCRC. The KEYNOTE-016 study showed that MSI-H mCRC patients responded to pembrolizumab treatment and showed a response rate of 40% and PFS of 78%[168]. The efficacy of pembrolizumab for the treatment of MSI-H mCRC was also validated in another phase I clinical trial [175]. The more recent trial, KEYNOTE-164, showed that when given in the second-line setting, pembrolizumab resulted in an objective response rate of 33%, PFS of 2.3 mo, and OS of 31.4 mo[176].

Table 3 Agents targeting mesenchymal epithelial transition factor receptor under clinical investigation for the treatment of colorectal cancer and metastatic colorectal cancer

Agent	Targeted molecule	Condition	Study phase	Clinical trial identifier
Savolitinib	MET	mCRC	Phase II	NCT03592641
Tivantinib	MET	mCRC	Phase I/II	NCT01075048
Onartuzumab	MET	CRC	Phase II	NCT01418222
Cabozantinib	MET/RET/VEGFR-2	CRC	Phase I	NCT02008383
		mCRC	Phase I	NCT03798626
		Refractory mCRC	Phase II	NCT03542877
Rilotumumab	HGF	KRAS wild-type mCRC	Phase I/II	NCT00788957

MET: Mesenchymal epithelial transition factor receptor; mCRC: Metastatic colorectal cancer; CRC: Colorectal cancer; VEGFR: Vascular endothelial growth factor receptor; HGF: Hepatocyte growth factor; RET: Rearranged during transfection.

The clinical benefit of PD-1 blockade in dMMR mCRC was also documented in the CheckMate 142 phase I trial of nivolumab in patients with refractory solid tumors, 14 of whom had mCRC. A durable complete response was achieved in one patient with mCRC, after receiving five doses of 3 mg/kg nivolumab[177]. This study led to the FDA approval of nivolumab for dMMR or MSI-H mCRC. Combined therapy with nivolumab and the CTLA-4 inhibitor ipilimumab produced durable clinical benefits and helped previously treated patients who had MSI-H or dMMR reach high PFS and OS rates [178,179]. The potential of PD-1 blockade using the single-agent dostarlimab was also evaluated in a very recent phase II study in patients selected for having dMMR stage II or III rectal adenocarcinoma. Administration of dostarlimab every three wk for six mo in twelve patients, who had not received chemoradiotherapy or undergone surgery, resulted in a clinical complete response in all patients with no evidence of progression or recurrence during the six to twenty-five mo follow-up[180]. Several preclinical studies are evaluating other potential immunotherapy agents. A novel antibody (LBL-007), recently characterized by Yu *et al*[181], targets lymphocyte activation gene 3 (LAG-3) expressed on activated T cells, natural killer cells, and B cells, and functions to negatively regulate these cells. This antibody was found to bind activated T cells and prevent LAG-3 binding to MHC class II molecules, blocking downstream signaling induction *in vitro*. *In vivo* results showed that treating mice bearing CRC with LBL-007 significantly delayed tumor growth and combining it with an anti-PD-1 antibody led to a more effective inhibition. Serum LBL-007 levels were high in monkeys injected with LBL-007 at 3, 10, or 30 mg/kg[181]. Another negative regulator of the immune system, T cell immunoglobulin and mucin domain 3, has been shown to be expressed in mCRC and plays an important role in cancer progression [181], and therefore might be a potential target for immunotherapy.

Pathways offering potential for targeted therapy

Several clinical trials have been initiated to evaluate the efficacy of agents targeting other pathways, yet no meaningful results have been presented so far. RO4929097 is a selective inhibitor of γ-secretase, a proteolytic enzyme that produces an activated intracellular Notch[182]. Notch is an attractive drug target as it is involved in CRC progression; however, a study of RO4929097 showed that no objective radiographic responses were observed and only a few mCRC patients had stable disease, although positive staining for intracellular Notch and its receptor was demonstrated in tissues[182]. A randomized phase II trial of vismodegib, a Hedgehog pathway inhibitor, reported no added benefit in combination with FOLFIRI or FOLFOX, and was instead associated with increased toxicity in mCRC patients[183]. The expression of morphogenetic protein 4 (BMP-4) has been shown to be upregulated in human CRC tissue and inhibition of BMP-4 by BMP type I receptor inhibitor, LDN-193189, induced apoptosis and inhibited tumor formation in mice injected with CRC cells[184]. The progress in the development of agents targeting TGF-β, Wnt, and ATP-binding cassette member B5 is still limited and needs further investigation[185-187]. Limitations in targeted therapy against these pathways are attributed to the existence of crosstalk between pathways, in addition to difficulty selecting patients, identifying predictive biomarkers, and specifically blocking targeted molecules. However, several clinical trials are investigating novel agents, which are summarized in Table 5.

BEATING RESISTANCE TO TARGETED THERAPY

Although multiple targeted therapy agents have demonstrated significant potency in mCRC patients, several challenges hinder the effectiveness of these therapies. Such therapies are associated with

Table 4 Agents targeting immune checkpoints under clinical investigation for the treatment of drug-resistant and metastatic colorectal cancer

Agent	Targeted molecule	Condition	Study phase	Clinical trial identifier
Camrelizumab	PD-1	Non-MSI-H/dMMR mCRC	Phase II	NCT04866862
		mCRC	Phase II	NCT03912857
Tislelizumab	PD-1	HER2-Positive Advanced CRC	Phase II	NCT05493683
Nivolumab	PD-1	Later-lines treatment of mCRC	Phase III	NCT05328908
		Advanced CRC	Phase I	NCT02991196
		Metastatic MSS CRC	Phase I	NCT03993626
		mCRC	Phase II	NCT04166383
Pembrolizumab (MK-3475)	PD-1	MSI-H/dMMR CRC	Phase III	NCT05239741
		mCRC	Phase III	NCT04776148
		MMR-proficient mCRC	Phase II	NCT03519412
		HER2-expressing mCRC	Phase II	NCT03631407
		HER2-expressing mCRC	Phase II	NCT05333809
PDR-001	PD-1/PD-L1	mCRC	Phase I	NCT03081494
		First-line mCRC	Phase I	NCT03176264
Toripalimab	PD-1/PD-L1	mCRC	Phase II	NCT03927898
Avelumab	PD-1/PD-L1	mCRC	Phase II	NCT03150706
		mCRC	Phase II	NCT03258398
Atezolizumab	PD-L1	mCRC	Phase III	NCT05425940
		mCRC	Phase III	NCT02788279
		First-line mCRC	Phase II	NCT02291289
		Refractory CRC	Phase II	NCT02873195
Relatlimab	LAG-3	Later-lines treatment of mCRC	Phase III	NCT05328908
Tremelimumab	CTLA-4	mCRC	Phase I/II	NCT03202758
		mCRC	Phase II	NCT03122509
		mCRC	Phase II	NCT03428126
		mCRC	Phase II	NCT03435107

PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; mCRC: metastatic colorectal cancer; MSI-H/dMMR: Microsatellite instability-high/mismatch repair deficient; MSS: Microsatellite stable; HER2: Human epidermal growth factor receptor 2; LAG-3: Lymphocyte activation gene 3; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4.

intrinsic and acquired resistance and a thorough understanding of resistance mechanisms is essential for developing effective drugs (Figure 3). For example, EGFR inhibitors are effective against *KRAS* WT mCRC but not *KRAS* mutated mCRC and there is a need for effective agents in this poor prognosis group. Several clinical trials have assessed the combination of VEGF and chemotherapy, but no attractive results have been shown[9,188].

Overcoming resistance to EGFR

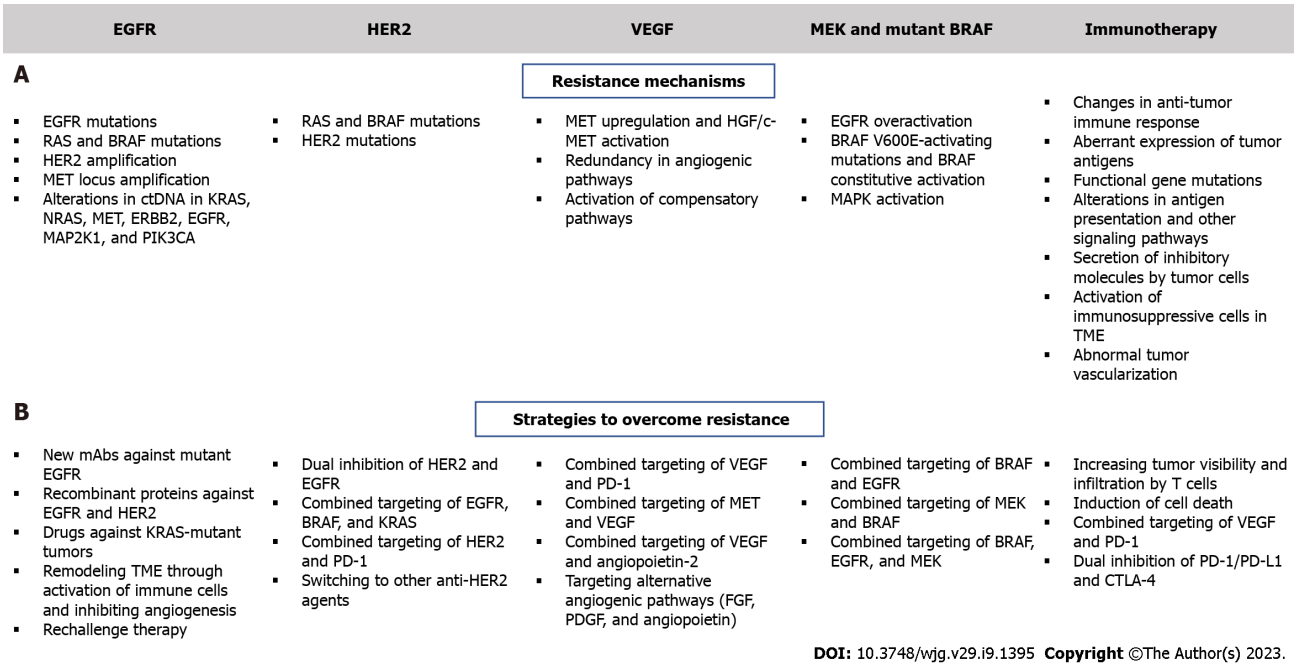
Administration of EGFR antibodies with MEK inhibitors has been tested in preclinical models, but clinical data are still limited[189]. Alterations in ctDNA in the following genes: *KRAS*, *NRAS*, *MET*, *ERBB2*, *FLT3*, *EGFR*, and *MAP2K1* have been identified in patients with primary or secondary resistance to EGFR inhibition[190]. Thus, determining the ctDNA profiles of patients with mCRC might help predict patient response[191]. Güttlein *et al*[192] recently tested *NRAS*, *KRAS*, and *BRAF* mutations in liquid plasma biopsies of patients with mCRC and reported a 12- and 4-mo median PFS of *RAS*/*BRAF* WT and *RAS*/*BRAF* mutated patients, respectively. The frequency of plasma mutations was highest for *KRAS* (34%). This study suggested that analysis of these mutations in the plasma of mCRC patients can be used to predict OS. The REVEAL study identified multiple actionable targets by performing NGS

Table 5 Agents targeting other pathways under clinical investigation for the treatment of drug-resistant and metastatic colorectal cancer

Agent	Targeted molecule	Condition	Study phase	Clinical trial identifier
CB-103	Notch	Resistant to oxaliplatin or irinotecan-based therapy advanced or mCRC	Phase I/II	NCT03422679
RO4929097	Notch	mCRC	Phase II	NCT01116687
WNT974	Wnt	BRAF-mutant mCRC	Phase I/II	NCT02278133
FOXY-5	Wnt	mCRC	Phase I	NCT02020291
LGK974	Wnt	BRAF mutant CRC	Phase I	NCT01351103
Vismodegib (GDC-0449)	Hedgehog	First-line therapy mCRC	Phase II	NCT00636610
		mCRC	Phase II	NCT00959647
LDE225	Hedgehog	mCRC	Phase I	NCT01576666
NIS793	TGF	Advanced CRC	Phase I	NCT02947165
LY3200882	TGF	Advanced chemotherapy -resistant CRC with an activated TGF-beta Signature	Phase I/II	NCT04031872
Ganitumab	IGF-1R	KRAS wild-type mCRC	Phase I/II	NCT00788957
		KRAS-mutant mCRC	Phase II	NCT00813605
Dalotuzumab (MK-0646)	IGF-1R	mCRC	Phase II	NCT00614393
Cixutumumab (IMC-A12)	IGF-1R	mCRC resistant to EGFR therapy	Phase II	NCT00503685

Wnt: Wingless-related integration site; mCRC: Metastatic colorectal cancer; TGF: Transforming growth factor; IGF-1R: Insulin growth factor receptor-1; EGFR: Epidermal growth factor receptor.

and transcriptional analysis of tumor and liquid biopsies during and after standard first-line chemotherapy treatment of patients with mCRC[193]. Differentially identified genes reported by this study were associated with EMT, ECM modulation, metabolism regulation, and several oncogenic pathways, such as PI3K/AKT and MAPK[193]. This study also reported the secreted phosphoprotein 1/osteopontin gene as a potentially druggable target whose inhibition also modulates the previously mentioned oncogenic pathways. Interestingly, the approach devised in this study aids in identifying mutations and transcriptional changes following first-line treatment, and thus can be used to predict novel resistance mechanisms and manage them by administering the appropriate targeted agents. Several clinical studies are underway to determine patient subsets who can benefit from anti-EGFR therapy[194,195]; however, sensitivity thresholds in PCR should be taken into consideration since they can affect the genotyping of *KRAS*, *NRAS*, *BRAF* and *PIK3C*. This would improve the selection of treatment for mCRC with anti-EGFR therapy, as shown by the ULTRA trial[196]. A prospective-retrospective cohort study documented that ctDNA *KRAS* tested using Digital PCR showed consistency with tumor tissues obtained from mCRC patients and predicted responses to EGFR inhibition[197]. Notably, recent studies have demonstrated that while left-sided *KRAS* WT mCRC should be preferentially treated with anti-EGFR agents, right-sided tumors might respond better to bevacizumab plus chemotherapy; however, optimization of treatment for these subsets of tumors is yet to be achieved[198-200]. Reversal strategies have emerged to overcome intrinsic resistance, and these include development of new EGFR inhibitors, combination of anti-EGFR with multitargeted inhibitors, development of small molecules that enhance the effect of anti-EGFR agents, and the implementation of metabolic regulators [201]. The development of EGFR mAbs that bind to mutated extracellular domains may enhance the efficacy of these treatments. A study involving CRC patients showed that MM-151, a mAb that binds to different regions of EGFR, significantly inhibits EGFR signaling and decreases mutations in ctDNA [202]. The FDA-approved anti-EGFR agent, necitumumab, was developed to bind to EGFR that harbors the most common cetuximab-resistant variant[203]. The first-in-class anti-EGFR non-overlapping mAbs mixture Sym004 has been documented to suppress mutant EGFR signaling in cetuximab-resistant cell lines and in xenograft models, contrary to cetuximab and panitumumab[204]. Interestingly, Sym004 is currently under clinical investigation for the treatment of mCRC (Table 1 and Figure 1). Notably, recombinant protein-based therapeutics have become an interesting therapeutic option for the treatment of resistant mCRC. A very recent study showed that $PEPD^{G278D}$, a recombinant human protein that induces the degradation of both EGFR and HER2, exerts strong anti-tumor activity and overcomes resistance to anti-EGFR therapy in CRC PDX[205]. As for patients with *KRAS*-mutant CRC, a fully



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Figure 3 Mechanisms of resistance to targeted therapy and strategies to overcome resistance in colorectal cancer. A: Resistance mechanisms; B: Strategies to overcome resistance. EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2; VEGF: Vascular endothelial growth factor; MEK: Mitogen-activated extracellular signal-regulated kinase; MAP2K1: Mitogen-Activated Protein Kinase 1; PI3KCA: Phosphoinositide 3-kinases catalytic subunit alpha; MAP2K1: Mitogen-activated protein kinase 1; HGF: Hepatocyte growth factor; MET: Mesenchymal epithelial transition; ERBB2: Erb-B2 Receptor Tyrosine Kinase 2; TME: Tumor microenvironment; FGF: Fibroblast growth factor; PDGF: Platelet-derived growth factor; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; CTLA-4: cytotoxic T lymphocyte-associated antigen 4.

humanized EGFR mAb (GC1118) showed significant inhibitory effects against *KRAS*-mutant CRC PDX [206] and hopes are now placed on the use of this novel compound for better targeting of these tumors.

Extrinsic resistance is mainly mediated by changes in the TME, specifically immune cells and CAFs, in addition to novel development of *KRAS* mutations and activation of angiogenesis[207,208]. Strategies to remodel the TME are usually beneficial to increase the efficacy of anti-EGFR antibodies and they may include activation of T cells and natural killer cells, suppression of CAFs, and inhibition of angiogenesis through VEGF blockade[201].

Interestingly, rechallenge and reintroduction strategies have been implemented in recent years and have been tested on patients with mCRC who have received an anti-EGFR therapy and whose treatment was halted[209]. Rechallenge refers to anti-EGFR re-treatment of *KRAS* WT mCRC patients who have initially received and benefited from first-line anti-EGFR therapy before disease progression and receiving a different treatment. Reintroduction refers to re-exposure after prior discontinuation of anti-EGFR therapy due to toxicity, intolerance, and other factors[209,210]. Very recently, Schulz *et al*[210] reported real-world evidence supporting the benefits of anti-EGFR treatment re-exposure in patients with mCRC, regardless of the reason for discontinuation of anti-EGFR therapy. The reintroduction or rechallenge of this treatment was associated with high OS and PFS[210], suggesting that the administration of more than one-line of treatment with anti-EGFR could be a promising tool to manage disease progression, given the limitations in the current treatment options.

Overcoming resistance to anti-HER2 therapy

Several strategies have been tested to combat resistance to anti-HER2 therapy (Figure 3). These include dual HER2 and EGFR inhibition in the first-line setting and increasing sensitivity to HER2 blockade following resistance to trastuzumab-based therapy[190,211]. Patients with HER2-amplified mCRC that harbor *RAS, BRAF,* or *PIK3CA* mutations show limited response to HER2 inhibitors[211], and therefore require a novel therapeutic strategy that would concomitantly block feedback loops involving *EGFR*, *BRAF*, and *KRAS* in mutated mCRC. In terms of the first strategy, several compounds are currently under clinical investigation and new drugs are being proposed as candidates to inhibit both molecules and improve efficacy of CRC targeted therapy, particularly in HER2-positive mCRC[212,213] (Table 1). In fact, HER2 amplification has been linked with resistance to EGFR inhibition[214] and thus, may serve as a biomarker for these treatment regimens. Moreover, combinations of HER2 and PD-1 inhibitors are also being investigated in HER2 expressing advanced CRC or mCRC (Table 6). As for patients with trastuzumab-refractory disease, a possible strategy would be to switch to another anti-HER2 agent. A novel antibody-drug conjugate (T-DM1) consisting of a mAb covalently linked to the cytotoxic agent DM1 has shown robust activity in patients with trastuzumab-resistant HER2-positive breast cancer

Table 6 Combination of targeted therapies under clinical investigation for the treatment of drug-resistant and metastatic colorectal cancer

Agents	Targeted molecule (s)	Condition	Study phase	Clinical trial identifier
Encorafenib + Binimetinib + Cetuximab	Wild type plus BRAF V600E and MEK, EGFR	Previously untreated BRAF-mutant mCRC	Phase II	NCT03693170
Tucatinib + Trastuzumab	HER2	First-line HER2-positive mCRC	Phase III	NCT05253651
Disitamab + Vedotin + Tislelizumab	HER2 and PD-1	HER2-positive advanced CRC	Phase II	NCT05493683
Vanucizumab + Bevacizumab	VEGF-A/angiopoietin-2 and VEGF	mCRC	Phase II	NCT02141295
Regorafenib + Nivolumab	VEGFR1/2/3 and PD-1	Later-lines treatment of mCRC	Phase III	NCT05328908
Lenvatinib + Pembrolizumab	VEGFR1/2/3 and PD-1	mCRC	Phase III	NCT04776148
Fruquitinib + Camrelizumab	VEGFR tyrosine kinase and PD-1	Non-MSI-H/dMMR mCRC	Phase II	NCT04866862
Disitamab + Vedotin + Pembrolizumab	HER2 and PD-1	HER2-expressing mCRC	Phase II	NCT05333809
Cobimetinib + Atezolizumab	MAPK and PD-L1	mCRC	Phase III	NCT02788279
Cetuximab + Vemurafenib	EGFR and mutated BRAF V600E	BRAF V600E Mutated Advanced CRC	Phase II	NCT03727763
Penpulimab + Anlotinib	PD-1 and VEGFR1/2/3	Refractory mCRC	Phase II	NCT04970914
Favezelimab	LAG-3 and PD-1	Previously treated metastatic PD-L1 positive CRC	Phase III	NCT05064059
MEN1611 + Cetuximab	PI3K and EGFR	mCRC	Phase I/II	NCT04495621
Encorafenib + Cetuximab + Pembrolizumab	BRAF V600E, as well as wild-type BRAF, EGFR, andPD-1	Previously untreated mCRC	Phase II	NCT05217446
RXC004 + Nivolumab	Porcupine (wnt activator) and PD1	RNF43 or RSPO aberrated, metastatic, MSS CRC after progression on SOC	Phase II	NCT04907539
Regorafenib + Pembrolizumab	VEGFR1/2/3PD1	Advanced or mCRC	Phase I/II	NCT03657641
Isatuximab + Atezolizumab	Epitope on CD38, and PD-L1	mCRC	Phase I/II	NCT03555149
Atezolizumab + Selicrelumab + Bevacizumab	PD-L1, CD40 antigen, and VEGF	mCRC	Phase I/II	NCT03555149
Atezolizumab + Idasanutlin	PD-L1 and MDM2	mCRC	Phase I/II	NCT03555149
Atezolizumab + Regorafenib	PD-L1 andVEGFR1/2/3	mCRC	Phase I/II	NCT03555149
Olaparib (MK-7339) + Bevacizumab	PARP and VEGF	Unresectable or mCRC	Phase III	NCT04456699
Nivolumab + Ipilimumab	PD-1 andCTLA-4	dMMR and/or MSI mCRC resistant to anti-PD1 monotherapy	Phase II	NCT05310643
Nivolumab + Ipilimumab	PD-1 and CTLA-4	dMMR and/or MSI mCRC	Phase II	NCT04730544
Surufatinib + Sintilimab	VEGFR1/2/3 and PD-1	Advanced MSS-Type CRC	Phase II	NCT04764006
Camrelizumab + Apatinib	PD-1 and VEGFR-2	Advanced CRC	Phase I/II	NCT04067986
Fruquintinib + Tislelizumab + Stereotactic ablative radiotherapy	VEGFR1/2/3 and PD-1	mCRC	Phase II	NCT04948034
Avelumab + Cetuximab + mFOLFOXIRI	PD-1/PD-L1 and EGFR	Unresectable mCRC	Phase II	NCT04513951
Geptanolimab (GB226) + Fruquintinib	PD-1 and VEGFR1/2/3	mCRC	Phase I	NCT03977090
Selinexor + Pembrolizumab	Exportin 1 and PD-1	Previously treated mCRC with RAS mutations	Phase II	NCT04854434
Panitumumab + Rilotumumab	EGFR and HGF	wild-type KRAS mCRC	Phase I/II	NCT00788957

Panitumumab + Ganitumab	EGFR and IGF-1R	wild-type KRAS mCRC	Phase I/II	NCT00788957

MEK: Mitogen-activated extracellular signal-regulated kinase; EGFR: Epidermal growth factor receptor; mCRC: Metastatic colorectal cancer; HER2: Human epidermal growth factor receptor 2; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; VEGF-A: Vascular endothelial growth factor-A; VEGFR: Vascular endothelial growth factor receptor; MSI-H/dMMR: Microsatellite instability-high/mismatch repair deficient; MSS: Microsatellite stable; MAPK: mitogen-activated protein kinases; LAG-3 Lymphocyte activation gene 3; PI3K: Phosphoinositide 3-kinases; RNF43: Ring Finger Protein 43; RSPO: R-spondin; SOC: Standard of Care; PARP: Poly ADP ribose polymerase; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; HGF: Hepatocyte growth factor; IGF-1R: Insulin growth factor receptor-1.

[215]. A clinical trial is currently evaluating the efficacy of this new compound in HER2-positive mCRC progressing after trastuzumab and lapatinib (Table 1).

Overcoming resistance to anti-VEGF therapy

The major mechanisms of resistance to anti-VEGF therapy are still not fully elucidated. Redundancy in angiogenic signaling pathways and compensation through activation of other pathways may contribute to this resistance (Figure 3). Several agents are currently under development for the purpose of improving anti-angiogenic therapy efficacy (Table 2). Importantly, it has recently been shown that the location of the primary tumor affects the choice of targeted therapy for the treatment of mCRC, whereby left-sided tumors benefit more than right-sided tumors from EGFR inhibition[198,200]. As mentioned before, combining anti-angiogenic agents with immune checkpoint inhibitors has been shown to restore vascular-immune crosstalk to establish a strong anti-tumor immune response[216]. In addition to VEGF/VEGFR, targeting alternative angiogenic pathways such as FGF, PDGF, and angiopoietins can inhibit VEGF-independent angiogenic pathways that are activated in response to VEGF blockade[217]. In mCRC patients, increased plasma levels of FGF, PDGF, and placental growth factor were linked to disease progression during bevacizumab-based therapy[217]. The clinical efficacy of the dual inhibition of VEGF-A and angiopoietin-2 using vanucizumab is still under phase II clinical trials, though with promising results[218] (Table 2). It is important to note that additional factors, including hypoxia and the limited blood supply restrict the delivery of drugs to the tumor site, resulting in resistance. In addition, cancer resistance to anti-VEGF therapy has been linked to activation of the HGF/c-MET pathway[219]. The latter activates key pathways involved in CRC metastasis and drug-resistance, including MAPK/ERK, STAT3, NF-κB, and PI3K/Akt[219]. Several MET inhibitors are being evaluated in the clinic for the purpose of blocking MET to overcome resistance to anti-VEGFR treatment (Table 3). This approach has produced effective results in other types of cancer, including advanced renal cell carcinoma[220]. Dual inhibition of MET and VEGFR2 using cabozantinib showed a strong anti-tumor effect in a preclinical CRC PDX model and the effect was greatest in tumors that possessed a mutation in the *PIK3CA* gene[146]. Several trials have been initiated to evaluate the efficacy of this compound in mCRC (Table 3).

Overcoming resistance to immunotherapy

Evading the immune system is an important hallmark of cancer, including CRC and is linked to immunotherapy and targeted therapy resistance[221]. Intrinsic resistance to immunotherapy is mainly conferred by changes in anti-tumor immune response, aberrant expression of tumor antigens, functional gene mutations, alterations in antigen presentation and other signaling pathways in tumor cells, in addition to secretion of inhibitory molecules by tumor cells[222] (Figure 3). Extrinsic mechanisms include activation of immunosuppressive cells in the TME and abnormal tumor vascularization[222]. One of the most effective strategies to deal with resistance to immunotherapy involves increasing tumor visibility and infiltration by T cells, through induction of immunogenic cell death by targeted agents and other therapies. The success of combining anti-angiogenic agents with immunotherapy has been shown in several cancers and is being evaluated in phase III clinical studies involving patients with advanced or metastatic and/or refractory CRC (Table 6). In addition, the efficacy of combining immune checkpoint inhibitors with chemokines that mediate the recruitment of T cells into the TME warrants investigation in mCRC. This could also be achieved by the administration of VEGF inhibitors that would normalize tumor vasculature and permit T-cell infiltration[223].

Enhancing the immune system function is also a good strategy to activate effector T cells and inhibit immunosuppressive immune cells. An emerging approach is the dual or combinatory inhibition of PD-1/PD-L1 and CTLA-4 to concomitantly block immune system inhibitory pathways and has shown promising results in preclinical[224,225] and clinical[226] (Table 6) models of mCRC. Ongoing trials are also addressing genomic and epigenetic alterations by evaluating the efficacy of anti-PD-1 agents in combination with VEGFR or CTLA-4 inhibitors in dMMR and/or MSI mCRC (Table 6).

Implementation of better preclinical models

The importance of preclinical models has been highlighted in the case of mCRC. The rapidly emerging role of patient-derived tumor samples may be considered one of the revolutionizing approaches to improve treatment strategies. Such samples can be propagated in mice to produce PDXs or in three-

dimensional cultures to produce patient-derived organoids (PDOs)[227-230]. These models are important for understanding and predicting treatment responses in drug-resistant CRC and mCRC. Molecular response predictors are usually identified in clinical trials by employing a statistically significant enrichment for a genetic mutation and correlating it with a clinical outcome in responsive and non-responsive patients. A major limitation of this approach is the inability to elucidate the mechanisms underlying this correlation and to validate whether these predictors influence response to treatment. Cancer cell line cultures have made it possible to gain insight into the functional processes; however, they do not recapitulate the *in vivo* structure, in addition to the genomic and functional heterogeneity of mCRC. Therefore, patient-derived models are ideal platforms with clinical fidelity and good reflection of disease diversity. These models are being used for target discovery and for characterization of response biomarkers to combat drug-resistance and to predict treatment response[228]. For example, PDX were used to validate the correlation between *KRAS* mutations in exon 2 and *de novo* resistance to EGFR inhibition and to identify HER2 as a potential target in cetuximab-resistant mCRC[89,231]. Additionally, these models were the first to identify *KRAS* exon 3 and 4 mutations as predictors of resistance to EGFR mAbs[89]. Both PDX and PDOs have clinical relevance; however, PDOs are easier to cultivate and are useful for high-throughput drug screening[232]. Subsequently, PDOs have been used to model CRC and study mechanisms of resistance. In addition, the newly emerging CRISPR/Cas9 genome-editing tool has been applied to introduce mutations in normal human colorectal organoids and has confirmed the role of these mutations in CSC maintenance, in addition to metastasis and resistance to therapy[233,234]. The association between *KRAS* mutation and lack of response to EGFR blockade has been also validated in organoids derived from mCRC[235]. Importantly, results from PDOs have been shown to recapitulate clinical response to targeted therapies, including cetuximab and regorafenib[236]. Notably, PDO-based drug screening has been used to improve the accuracy and effectiveness of precision medicine, paving the way for PDO-based personalized therapy[237]. CRC PDOs can be also used to identify patients that benefit from a specific targeted therapy.

CONCLUSION

Given the high molecular heterogeneity associated with CRC, different mechanisms of resistance may develop. A multi-targeted approach to therapy and the use of combination targeted therapy as a first-line treatment, rather than after the patients demonstrate drug-resistance and progress on treatment, have been an active area of research based on the efficacy of these strategies in preclinical models. Several clinical trials have investigated the efficacy of combination therapies targeted at two or three pathways; however, the high toxicity levels associated with these therapies is a limitation to bear in mind as it represents a critical challenge to the development of effective therapies for the treatment of drug-resistant and mCRC. Nevertheless, data from clinical studies are showing promising signs of efficacy. This has been made possible through targeting adaptive feedback pathways and the discovery and implementation of predictive biomarkers for targeted therapy, which are critical in identifying patients that could benefit from combination targeted therapy. Biomarker detection computational algorithms and tools are being designed for this purpose and should be followed by clinical validation and approval. Importantly, personalized treatment could be developed to promote survival and prognosis of CRC patients without causing adverse events. With the advancement of NGS and genome profiling, it has been possible to decipher predictive responses to anti-cancer treatments and to select the appropriate treatment for each individual, depending on the genetic characteristics and clinical tumor features. Strategic planning of treatment regimens is essential to enhance the effectiveness of targeted agents and to decrease the possibility of side effects. Conjugation of inhibitory molecules using Nanoparticle technology is an attractive approach in this case. Nanoparticles are being used for the targeted delivery of drugs to the affected tissues and optimization methods can be applied to increase their uptake efficiency.

Other tools that could help improve personalized medicine include the triphasic enhanced computed tomography radiomics signature that was recently tested by Cao *et al*[238] and has been shown to be effective in predicting CRC MSI status with 0.837 and 0.821 accuracy and sensitivity, respectively. Moreover, whole genome sequencing, multi-region whole exome sequencing, simultaneous single-cell RNA-sequencing, and single-cell targeted cDNA Sanger sequencing are being used to obtain single-cell genomic and transcriptomic landscapes of adjacent normal tissues, primary tumors, and metastatic tumors[239], which could also improve individualized treatment.

Given the importance of the gut microbiota in the progression of CRC, microbiome profiles can be integrated with other genomic and epigenomic profiles to enhance personalized targeted therapies against CRC, resulting in better clinical outcomes. Nonetheless, this adds another level of complexity to the application of this approach. Interestingly, modification of the gut microbiota through targeted inhibition of pathogenic bacteria can be used to prepare patients for CRC treatment by augmenting the host immune system.

Changes in mutations or transcription should be monitored during administration of treatment, in addition to changes in immune responses and inflammatory molecules that can influence the choice of

treatment. These immune signatures may be indispensable for improving clinical outcome. Interestingly, it has been reported that the peripheral blood repertoire of T cell receptor changes during the course of chemotherapy in patients with mCRC, and thus could have a prognostic value[12].

In summary, the application of personalized medicine requires the integration of tumor mutations and epigenetic modifications, TME gene expression, host immune proficiency, and their changes during disease progression and treatment. The constant search for novel targets involved in drug-resistance and metastasis will lead to the identification of interesting molecular traits that can be modulated using biomarker-driven treatments to overcome resistance to therapy.

FOOTNOTES

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REVIEW

Clinical impact of artificial intelligence-based solutions on imaging of the pancreas and liver

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Abstract

Artificial intelligence (AI) has experienced substantial progress over the last ten years in many fields of application, including healthcare. In hepatology and pancreatology, major attention to date has been paid to its application to the assisted or even automated interpretation of radiological images, where AI can generate accurate and reproducible imaging diagnosis, reducing the physicians' workload. AI can provide automatic or semi-automatic segmentation and registration of the liver and pancreatic glands and lesions. Furthermore, using radiomics, AI can introduce new quantitative information which is not visible to the human eye to radiological reports. AI has been applied in the detection and characterization of focal lesions and diffuse diseases of the liver and pancreas, such as neoplasms, chronic hepatic disease, or acute or chronic pancreatitis, among others. These solutions have been applied to different imaging techniques commonly used to diagnose liver and pancreatic diseases, such as ultrasound, endoscopic ultrasonography, computerized tomography (CT), magnetic resonance imaging, and positron emission tomography/CT. However, AI is also applied in this context to many other relevant steps involved in a comprehensive clinical scenario to manage a gastroenterological patient. AI can also be applied to choose the most convenient test prescription, to improve image quality or accelerate its acquisition, and to predict patient prognosis and treatment response. In this review, we summarize the current evidence on the application of AI to hepatic and pancreatic radiology, not only in regard to the interpretation of images, but also to all the steps involved in the radiological workflow in a broader sense. Lastly, we discuss the challenges and future directions of the clinical application of AI methods.

Key Words: Artificial intelligence; Machine learning; Deep learning; Imaging; Liver; Pancreas

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Core Tip: The gastroenterology field is changing with the application of artificial intelligence (AI) solutions capable of assisting and even automating the interpretation of radiological images (ultrasound, endoscopic ultrasound, computerized tomography, magnetic resonance imaging, and positron emission tomography), generating accurate and reproducible diagnoses. AI can further be applied to other steps of the radiological workflow beyond image interpretation, including test selection, image quality improvement, acceleration of image acquisition, and prediction of patient prognosis and outcome. We herein discuss the current evidence, challenges, and future directions on the application of AI to hepatic and pancreatic radiology.

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INTRODUCTION

Malignant tumors of the liver and pancreas are among the most common and lethal types of cancer. According to the recent GLOBOCAN 2020 data[1], liver and pancreas are the 6th and 12th most common sites for primary cancer, with 905677 and 495773 new cases in 2020, respectively. However, they also represent the 3rd and 7th neoplasia with the highest mortality, causing 830180 and 466003 deaths worldwide in 2020, respectively. If taken combined, cancer at the liver or pancreas thus represents the 5th most incident and the second most lethal one.

Cancer at these locations account for almost as many deaths as cases. Five-year survival rates are 20% for liver cancer[2] and as low as 11% for pancreatic cancer[3], making them two of the cancer sites with the poorest prognosis. Other non-oncologic diseases affecting these organs are also highly prevalent, such as diffuse liver disease, including chronic liver disease, which affects tens of millions of people globally and represents a substantial socioeconomic burden[4].

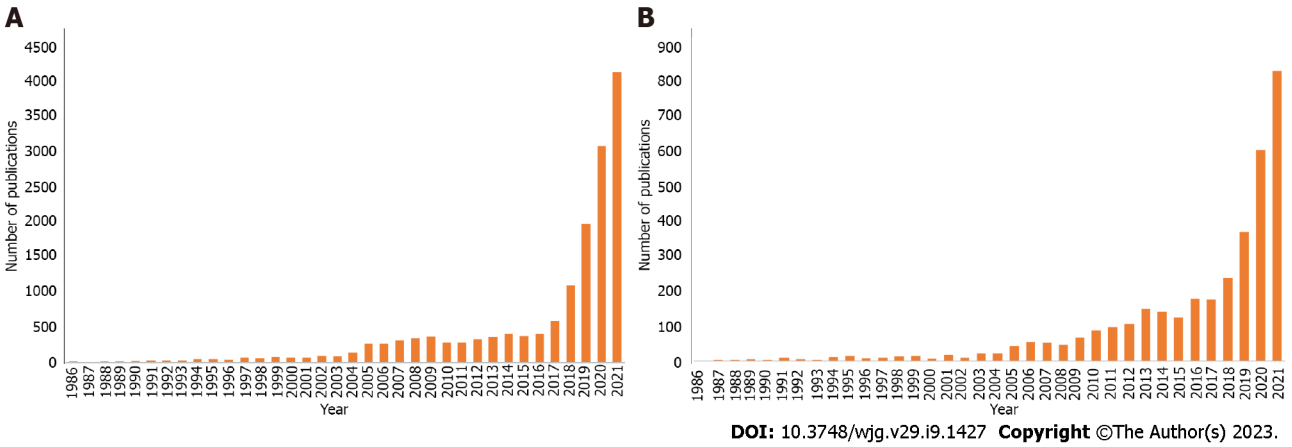
Clinical outcomes of patients with these types of disease depend on a variety of factors, including stage and disease extension as assessed by imaging, and correct election of treatment. Thus, there is an unmet need for new tools capable of assisting specialists in early detection, characterization, and management of these diseases.

In recent years, artificial intelligence (AI) has shown promise in different areas of healthcare. The evaluation of medical images by machine learning (ML) approaches is a leading research field which, in gastroenterology, has applications in automatic analysis of different types of images, such as radiology, pathology, and endoscopy studies[5].

The first applications of AI to radiology have been dominated by anatomic locations such as the brain or the breast. Image analysis of abdominal organs, such as the liver and pancreas, are more challenging. Magnetic resonance imaging (MRI) in these locations, especially at 3 T, is prone to motion and field inhomogeneity artifacts, which are aggravated by larger fields of view[6]. As a result, advances in automatic analyses of abdominal images have gathered comparatively less attention. Nonetheless, the application of AI in liver and pancreas imaging is also gaining increasing interest (Figure 1). The goal of this review is to summarize the current experience on the use of AI to assist radiologists in their workflow, acquisition, and interpretation of medical images of the liver and pancreas.

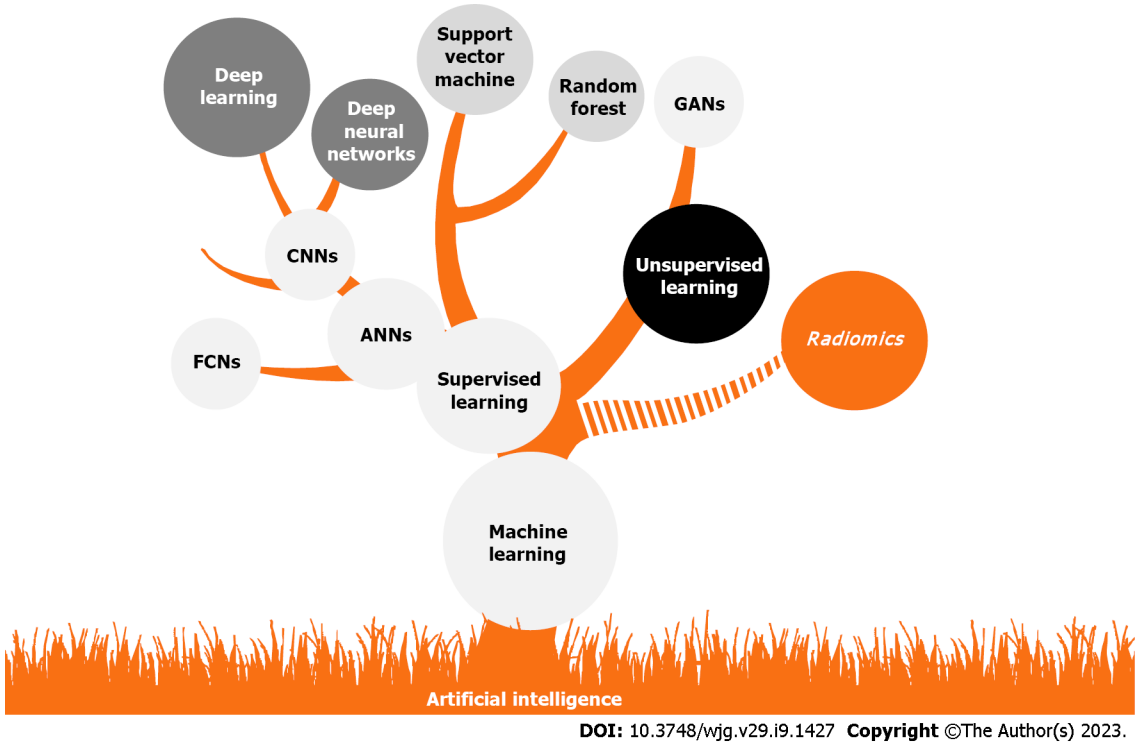
AI IN RADIOLOGY: BASIC PRINCIPLES

Artificial intelligence is expected to revolutionize the medical field, deeply impacting the hospital and clinical settings by potentially improving diagnostic accuracy, treatment delivery, and allowing a more personalized medical care[7]. Radiology will arguably be one the most changed areas of medicine because of AI implementation in its workflows, as the information-rich images generated in this field are an excellent source of data for the development of AI algorithms. Broadly, the term AI refers to a wide range of technologies and computing processes capable of imitating human intelligence to extract information from input data to solve a problem. This rapidly evolving area has a vocabulary of its own (Figure 2) that can be daunting to those not familiar with the field, including terms that are oftentimes



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Figure 1 PubMed results by year using the search terms. A and B: Artificial intelligence radiology (top) and artificial intelligence AND (liver OR pancreas) (bottom).



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Figure 2 Relation between artificial intelligence and related subdisciplines, neural network architectures, and/or techniques. ANN: Artificial neural network; FCN: Fully convolutional network; CNN: Convolutional neural network; GAN: Generation adversarial network.

used as synonyms to AI, such as ML.

ML is actually a subset of AI consisting of those methods capable of training a computer system to perform a given task based on provided information or experience without explicit programming, thus conferring machines the ability to learn[8]. The aim of ML is to predict an output based on a given input (a training dataset). Common ML applications in radiology include classification, image segmentation, regression, and clustering[9]. ML can be sub-divided into supervised and unsupervised learning[10]. In supervised learning, the most common type used in medical research, the algorithm is trained with labeled examples (*i.e.*, the correct output for these training data, known as ground truth, is already known). Among the methods employed in supervised learning, random forest (RF), and specially, support vector machine (SVM), are powerful algorithms frequently used for the classification of images [7], including image segmentation. Conversely, in unsupervised learning, the ground truth is not known, as the algorithm is trained with unlabeled data that must be classified by the algorithm itself.

Artificial neural networks (ANNs), named after their brain-inspired structure and functioning process, can be trained *via* both supervised and unsupervised ML. In these ANNs, input information flows through a variable number of layers composed of artificial neurons, joined by weighted

connectors, that process the data to obtain an output that matches the ground truth as closely as possible. Generative adversarial networks (GANs) are an example of ANN trained *via* unsupervised learning. GANs include two networks: One which creates new data based on input examples (*i.e.*, generator), and one which distinguishes between different types of data (*i.e.*, discriminator)[11]. These networks can be used to produce realistic, synthetic images as a strategy for data augmentation[12]. Similarly, the structure of convolutional neural networks (CNNs), a type of ANN specially designed for computer vision tasks, is based on that of the animal visual cortex. Typically used in image recognition and classification, in CNNs the input information is filtered and analyzed through a convolutional layer, and the size of the resulting image is subsequently reduced by a pooling layer. This two-step process will be repeated as many times as layers integrate the CNN, with a final step in which an ANN will classify the image (Figure 3). Fully convolutional networks (FCNs, a type of ANN that only performs the convolution step) are the basis for U-net, a modified architecture that consists of a contracting path including several convolutional and pooling layers to capture context, followed by a symmetric expanding path including a number of up-sampling and convolutional layers to enable accurate localization. U-net is a popular network for the development of automatic segmentation algorithms, as it requires relatively small datasets for algorithm training[13].

Deep learning (DL) is a section of ML that utilizes multi-layered ANNs, referred to as deep neural networks (DNN), allowing the exploration of more complex data[14]. DL algorithms are gaining attention and raising considerable enthusiasm thanks to their scalability, easy accessibility, and ability to extract relevant information from the data without further indications other than input data. The recently developed nnU-Net, a publicly available DL-based segmentation tool capable of automatically configuring itself, has set a new state-of-the-art standard thanks to the systematization of the configuration process, which used to be a manual, complicated, and oftentimes limited task in previous approaches[15]. Improvement of the computational resources and the development of cloud technologies are also contributing to the application of DL architectures in a wide variety of research fields beyond medicine[14].

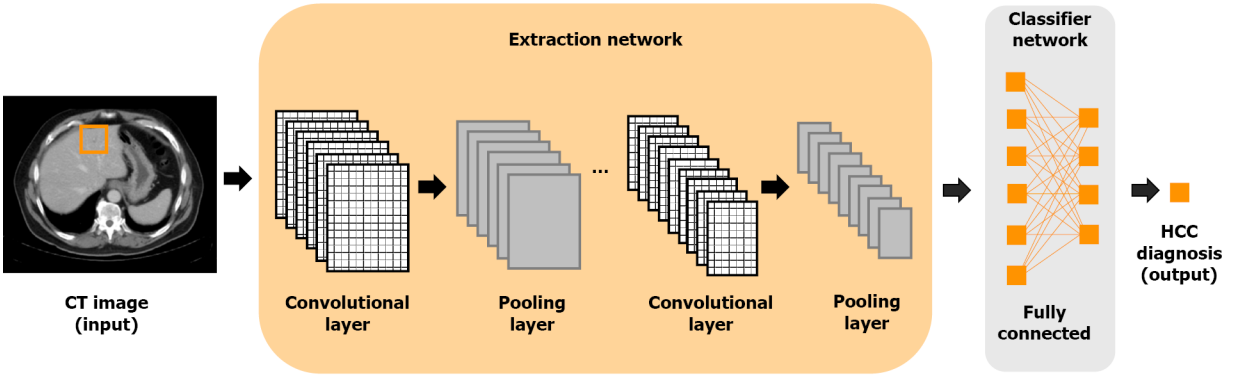
Closely related to the development of AI, the term radiomics refers to the computational extraction (*via* ML and DL algorithms) of quantitative data from radiological image features[16]. A particularly useful and valuable application of radiomics is the analysis of radiologic textures, defined as the differences in the grayscale intensities in the area of interest, which have been associated with intratumor heterogeneity[17] and that can potentially provide clinically relevant information that otherwise would remain unknown.

IMAGE ACQUISITION

The ultimate aim of computerized tomography (CT) and MRI is to unveil clinically relevant information; thus, the importance of this information relies heavily on the quality of the image. For CT, radiation dose is a parameter as important as image quality, and both are closely related to acquisition and reconstruction times. Iterative reconstruction (IR) algorithms[18] are the current technique of choice to transform the raw data into a 3D volume presented as an anatomical image. These algorithms generate an image estimate that is projected forward into a synthetic sinogram; subsequently, this image estimate is iteratively rectified by comparison with the real raw data sinogram until the algorithm's predefined endpoint condition is met, resulting in enhanced image quality and thus allowing an important dose reduction[19]. DL reconstruction algorithms (DLR) are currently being developed with the aim to further improve image quality, therefore further reducing radiation doses. Compared to IR algorithms, DLR algorithms trained with low-dose data offer an improved signal-to-noise (SNR) ratio, as demonstrated by the U-net-based CNN developed by Jin *et al*[20], thus facilitating the detection of lesions of any kind and the increased use of low-dose imaging. Currently, there are two commercially available DLRs: TrueFidelity (GE Healthcare, Chicago, IL, United States) and AiCE (Canon Medical Systems, Otawara, Japan). Akagi *et al*[21] employed AiCE in their study and reported improved contrast-to-noise ratio and image quality in CT images, compared to images created with a hybrid IR algorithm. Although the preliminary results are exciting, further validation for these DLR algorithms is required, and real dose reduction in the clinical setting has yet to be demonstrated.

An important setback of MRI is the long acquisition time, forcing the patient to lay still for a relatively long period and with any movement affecting the quality of the image. One way to reduce acquisition time is compressed sensing, based on the idea that if signal information is only present in a small portion of pixels, that sparsity can be used to reconstruct a high-definition image from considerably less collected data (undersampling). Kaga *et al*[22] evaluated the usefulness of the Compressed SENSE algorithm (Philips, Amsterdam, The Netherlands) in MRI of the abdomen using diffusion weighted images (DWIs) and reported a significantly improved image noise and contour of the liver and pancreas and higher apparent diffusion coefficient values, thus offering superior image quality compared to parallel imaging (PI)-DWI[22].

AI applications have also been designed to automate MRI and CT protocol selection with the aim to standardize workflows and increase effectiveness in the radiology setting. The selection of an



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Figure 3 Diagram of a convolutional neural network used for the classification of a focal liver lesion in a computerized tomography image. HCC: Hepatocellular carcinoma; CT: Computed tomography.

appropriate imaging protocol requires taking into account factors including the type of procedure, clinical indication, and the patient's medical history. The increasing incorporation of electronic medical records and other digital content has opened opportunities for the application of natural language processing (NLP) methods to extract structured data from unstructured radiology reports. López-Úbeda *et al*[23] developed an NLP-based classification system for automated protocol assignment that offered an overall accuracy of 92.25% for the CT and 86.91% for the MRI datasets. This system has already been successfully implemented and is currently in use at the HT Médica centers.

Information about the respiration of the patient can be used for functional studies, overall monitoring, or motion compensation during the performance of an MRI. Typically, breathing is measured *via* belts or nasal sensors that can potentially alter the raw MRI data. Using adaptive intelligence, the laser-based VitalEye system (Philips) registers a contactless continuous respiratory signal, with up to 50 body locations analyzed simultaneously and in real time, thus producing a more robust respiratory trace compared to traditional respiratory belts[24]. Moreover, as soon as the patient is lying on the table, the BioMatrix Respiratory Sensors (Siemens AG, Munich, Germany) embedded in the spinal coil produce a local magnetic field that changes with the variation of lung volume during breathing. These changes are registered, and the breathing pattern is integrated to optimize image quality[25]. By standardizing and accelerating the workflow, these advances allow technicians and radiologists to concentrate on the patient.

IMAGE ANALYSIS

Segmentation of liver and pancreas

Image analysis has experimented a huge progression with the advent of AI, and especially with DL, reaching state-of-art performances in many biomedical image analysis tasks[26-28] (Table 1). Among them, segmentation is one of the most important in radiology. For instance, accurate pancreas segmentation has applications in surgical planning, assessment of diabetes, and detection and analysis of pancreatic tumors[29]. Another key application of organ and lesion contouring is treatment volume calculation for radiotherapy planning. However, boundary delimitation of anatomical structures in medical images remains a challenge due to their complexity, particularly in the upper abdominal cavity, where there are constant changes in the position of the different organs with the respiratory cycle, as well as the occurrence of anatomical variants and pathological changes of organs[30].

The intersubject variability and complexity of the pancreas make segmentation of this organ a demanding task. Segmentation of pancreatic cancer lesions is particularly challenging because of their limited contrast and blurred boundaries against the background pancreatic parenchyma in CT and MR images[31]. In addition, other factors such as body mass index, visceral abdominal fat, volume of the pancreas, standard deviation of CT attenuation within pancreas, and median and average CT attenuation in the immediate neighborhood of the pancreas may affect segmentation accuracy[29,32].

These problems lead to high segmentation uncertainty and inaccurate results. To tackle these problems, Zheng *et al*[33] proposed a 2D, DL-based method that describes the uncertain regions of pancreatic MR images based on shadowed sets theory. It demonstrated high accuracy, with a dice similarity coefficient (DSC) of 73.88% on a cancer MRI dataset and 84.37% on the National Institutes of Health (NIH) Pancreas dataset (which contains 82 CT scans of healthy pancreas), respectively. The same authors reported[34] a more sophisticated 2.5D network that benefits from multi-level slice interaction. They surpassed state-of-art performances in the NIH dataset, with a DSC of 86.21% ± 4.37%, sensitivity of 87.49% ± 6.38%, and a specificity of 85.11% ± 6.49%.

Table 1 Works proposed for automated image analysis

Image analysis	Anatomical area	Modality	AI model	Ref.
Segmentation	Pancreas	MRI	CNN	[33,34,110]
			UDCGAN	[111]
			3D-Unet	[112]
	Liver	CT	SSC (no AI)	[36]
			PA (Atlas-no AI)	[39]
		MRI	CNN	[37,38,42,113]
			GAN	[43]
Registration	Liver	CT, MRI	CNN	[47]
			SG-DIR (no AI)	[48]
			Cycle-GAN + UR-Net	[46]
		4D-MRI	Non-rigid	[49]

MRI: Magnetic resonance imaging; CT: Computerized tomography; 4D-MRI: Four-dimensional magnetic resonance imaging; CNN: Convolutional neural network; UDCGAN: U-Type densely connected generation adversarial network; SCC: Sparse shape composition; AI: Artificial intelligence; PA: Probabilistic atlas; GAN: Generation adversarial network; SG-DIR: Structure-guided deformable image registration; UR-Net: Unsupervised registration network.

The liver is also a popular target for automated segmentation algorithms. Automatic segmentation of this organ is regarded as somewhat less challenging than that of the pancreas, with reported DSC scores typically in the > 0.90 range[35].

Li *et al*[36] presented a liver segmentation method from abdominal CT volumes for both healthy and pathological tissues, based on the level set and sparse shape composition (SSC) method. The experiments, performed using public databases SILVER07 and 3Dicardb, showed good results, with mean ASD, RMSD, MSD, VOE, and RVD of 0.9 mm, 1.8 mm, 19.4 mm, 5.1%, and 0.1%, respectively. Moreover, Winther *et al*[37] used a 3D DNN for automatic liver segmentation along with a Gd-EOB-DTPA-enhanced liver MR images dataset. Results show an intraclass correlation coefficient (ICC) of 0.987, DSC of 96.7% ± 1.9%, and a Hausdorff distance of 24.9 mm ± 14.7 mm compared with two expert readers who corresponded to an ICC of 0.973 and a DSC of 95.2% ± 2.8%. Finally, Mohagheghi *et al*[38] used a CNN but further incorporated prior knowledge. The model learnt the global shape information as prior knowledge by using a convolutional denoising auto-encoder; then, this knowledge was used to define a loss function and combine it with the Dice loss in the main segmentation model. This model with prior knowledge improved the performance of the 3D U-Net model and reached a DSC of 97.62% segmenting CT images of the Silver07-liver dataset.

Organ segmentation is even more challenging in pediatric patients studied with CT, as it is acquired at a low dose to minimize harmful radiation to children, thus having a lower SNR. Nakayama *et al*[39] proposed a liver segmentation algorithm for pediatric CT scans using a patient-specific level set distribution model to generate a probabilistic atlas, obtaining a DSC index of 88.21% in the segmentation. This approach may be useful for low dose studies in general, *i.e.*, also in the adult population.

Algorithms for automatic segmentation of the liver using MR images have proven equally efficient. For instance, Bobo *et al*[40] used a 2D FCN architecture to segment livers on T2-weighted MR images with a DSC score of 0.913. In a recent paper, Saunders *et al*[41] systematically analyzed the performance of different types of MR images in the training of CNN for liver segmentation, using a 3D U-net architecture. Water and fat images outperformed other modalities, such as T2* images, with a DSC of 0.94.

Conversely, high-quality automatic segmentation of liver lesions is not an easy task, since the low contrast between tumors and healthy liver parenchyma in CT images, their inhomogeneity, and its complexity pose a challenge for liver tumor segmentation. In addition, motion-induced phase errors due to peristaltic and respiratory movements negatively affect image quality and assessment of liver lesions in MR images. A 3D CNN was used by Meng *et al*[42] where a special three-dimensional dual path multiscale convolutional neural network (TDP-CNN) was designed for liver tumor segmentation. Results achieved in the LiTS public dataset were a DSC of 68.9%, Hausdorff distance of 7.96 mm, and average distance of 1.07 mm for liver tumor segmentation and a DSC of 96.5%, Hausdorff distance of 29.162 mm, and average distance of 0.197 mm for liver segmentation. A different approach for liver tumor segmentation was proposed by Chen *et al*[43]. In this work, an adversarial densely connected network algorithm was trained and evaluated using the Liver Tumor Segmentation challenge dataset. Results revealed an average Dice score of 68.4% and ASD, MSD, VOE, and RVD of 21 mm, 124 mm,

0.46%, and 0.73%, respectively.

Automatic contouring of hepatic tumor volumes has also been reported using CT scans, a modified SegNet CNN[44], and dynamic contrast enhanced (DCE)-MRI images in a U-net-like architecture[45], for example.

Some medical imaging vendors incorporate solutions for liver segmentation and hepatic lesion characterization integrated in the proprietary radiologist's workflow. For instance, the Liver Analysis research application from Siemens Healthcare (Erlangen, Germany) aims to provide AI support for liver MRI and CT reading. The tool includes DL-based algorithms for automatic segmentation of the whole liver, functional liver segments, and other abdominal organs like the spleen and kidneys (Figure 4A). It also features an AI method to automatically detect and segment focal liver lesions, providing lesion diameter, volume, and 3D contours (Figure 4B).

Registration

Medical image registration seeks to find an optimal spatial transformation that best aligns the underlying anatomical structures. Medical image registration is used in many clinical applications such as image guidance systems (IGS), motion tracking, segmentation, dose accumulation, image reconstruction, *etc*[28]. In clinical practice, image registration is a major problem in image-guided liver interventions, especially for the soft-tissues, where organ shape changes occurring between pre-procedural and intra-procedural imaging pose significant challenges[46]. Schneider *et al*[47] showed how semi-automatic registration in IGS may improve patient safety by enabling 3D visualization of critical intra- and extra-hepatic structures. A novel IGS (SmartLiver) offering augmented reality visualization was developed to provide intuitive visualization by using DL algorithms for semi-automatic image registration. Results showed a mean registration accuracy of 10.9 mm ± 4.2 mm (manual) *vs* 13.9 mm ± 4.4 mm (semi-automatic), hence significantly improving the manual registration. Kuznetsova *et al*[48] assessed the performance of structure-guided deformable image registration (SG-DIR) relative to rigid registration and DIR using TG-132 recommendations for 14 patients with liver tumors to whom stereotactic body radiation therapy (SBRT) was applied. The median DSC for rigid registration was 88% and 89% for DIR, and 90% for both SG-DIR using liver contours only and using liver structures along with anatomical landmarks. However, most of the existing volumetric registration algorithms are not suitable for the intra-procedural stage, as they involve time-consuming optimization. In the report by Wei *et al*[46], a fast MR-CT image registration method was proposed for overlaying pre-procedural MR (pMR) and pre-procedural CT (pCT) images onto an intra-procedural CT (iCT) image to guide thermal ablation of liver tumors. This method, consisting of four DL-based modules and one conventional ANTs registration module, showed higher Dice ratios (around 7% improvement) over tumors and compatible Dice ratios over livers. However, its main advantage was the computational time cost of around 7 s in the intra-procedural stage, which is only 0.1% runtime in the conventional way (*i.e.*, ANTs).

Treatment planning concepts using the mid-ventilation and internal-target volume concept are based on the extent of tumor motion between expiration and inspiration. Therefore, four-dimensional (4D) imaging is required to provide the necessary information about the individual respiration-associated motion pattern. Weick *et al*[49] proposed a method to increase the image quality of the end-expiratory and end-inspiratory phases of retrospective respiratory self-gated 4D MRI data sets using two different non-rigid image registration schemes for improved target delineation of moving liver tumors. In the first scheme, all phases were registered directly (dir-Reg), while in the second next neighbors were successively registered until the target was reached (nn-Reg). Results showed that the Median dir-Reg coefficient of variation of all regions of interest (ROIs) was 5.6% lower for expiration and 7.0% lower for inspiration compared with nn-Reg. Statistically significant differences were found in all comparisons.

DIAGNOSIS

Two decades ago, the methods proposed for ML-based diagnosis required manually extracting the features from the images. This tedious step has been partially relieved with the irruption of CNNs. However, techniques such as radiomics are still in use to try to improve the performance of novel AI methods for medical diagnosis. Radiomics concerns the high throughput extracting of comprehensible features from radiological images that can be further analyzed by ML algorithms for classification or regression tasks. In this section, different methods proposed for liver and pancreas imaging diagnosis are reviewed (Table 2).

Liver-CT

Starting with chronic liver disease, Choi *et al*[50] presented a CNN model for staging liver fibrosis from contrast-enhanced CT images. Before using the CT image as input for the CNN, the liver is segmented. The testing dataset included 891 patients and the CNN achieved a staging accuracy of 79.4% and an AUC of 96%, 97%, and 95% for diagnosing significant fibrosis, advanced fibrosis, and cirrhosis, respectively. A different approach was proposed by Nayak *et al*[51], where SVM was used instead of CNN for aiding in the diagnosis of cirrhosis and hepatocellular carcinoma (HCC) from multi-phase

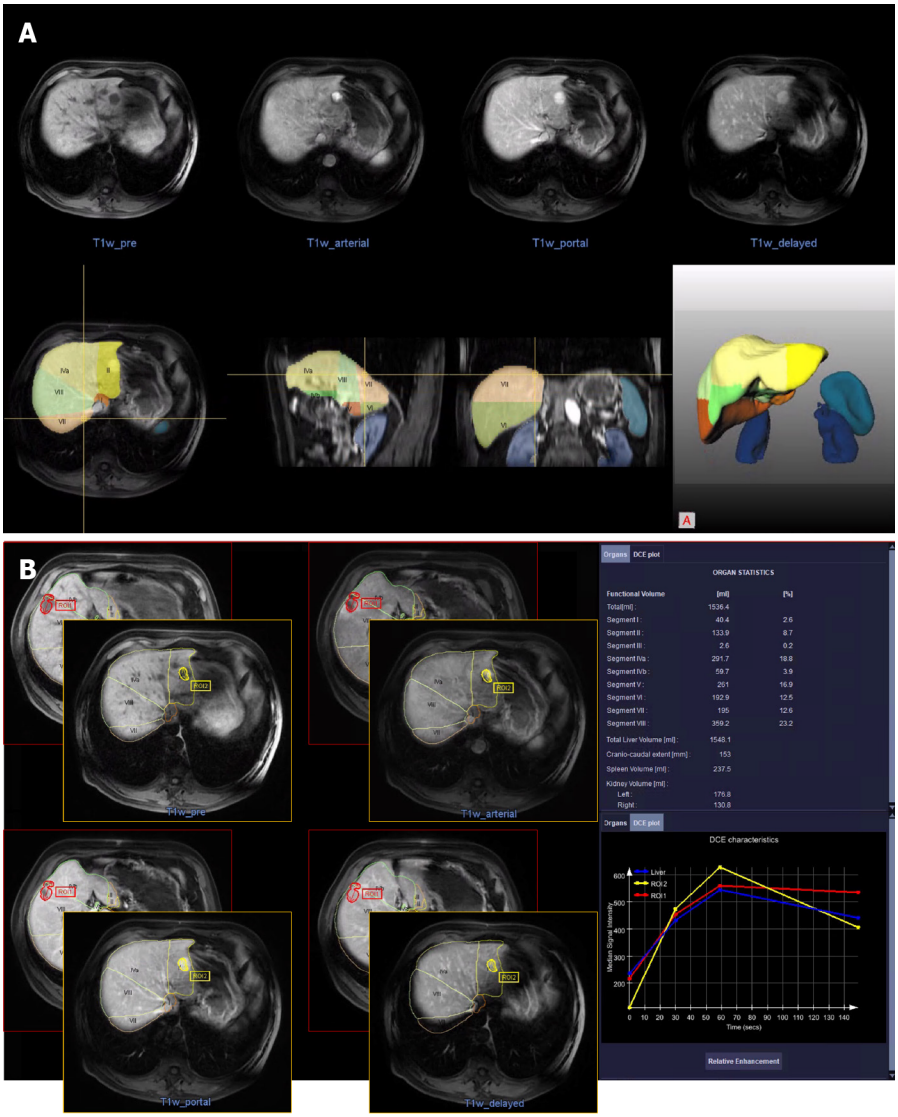
Table 2 Summary of works based on artificial intelligence for automated diagnosis of pancreas and hepatobiliary system diseases

Anatomical area	Modality	AI model	What is diagnosed?	Ref.
Liver	Scintiscan	ANN	Chronic hepatitis and cirrhosis	[114]
	CT	ANN	HCC, intra-hepatic peripheral cholangiocarcinoma, hemangioma, metastases	[52]
		CNN	HCC, malignant liver tumors, indeterminate mases, hemangiomas, cysts	[53]
			Liver fibrosis	[50,115]
		SVM	Cirrhosis and HCC	[51]
			Malignant liver tumors	[54]
		KNN, SVM, RF	HCC	[116]
	MRI	CNN	HCC	[55]
			Simple cyst, cavernous hemangioma, FNH, HCC, ICC	[56,57]
		Extremely randomized trees	Adenomas, cysts, hemangiomas, HCC, metastases	[58]
	US	PNN	Benign and malignant focal liver lesions	[65]
		SVM	Fatty liver	[68]
			HCC	[66]
		CNN	Focal liver lesions: Angioma, metastasis, HCC, cyst, FNH	[67]
			Liver fibrosis stages	[69]
Biliary system	MRI	ANN	Cholangiocarcinoma	[59,60]
			Lymph node status in ICC	[117]
Pancreas	CT	Hybrid SVM-RF	Pancreas cancer	[76]
		SVM	Serous cystic neoplasms	[72]
		CNN	IPMN, mucinous cystic neoplasm, serous cystic neoplasm, solid pseudopapillary tumor	[73]
	MRI	SVM	IPMN	[78]
	US	ANN	Chronic pancreatitis, pancreatic adenocarcinoma	[81]
		CNN	Malignancy in IPMN	[82]
			Autoimmune pancreatitis, pancreatic ductal adenocarcinoma, chronic pancreatitis	[83]

CT: Computerized tomography; MRI: Magnetic resonance imaging; US: Ultrasound; ANN: Artificial neural network; CNN: Convolutional neural network; SVM: Support vector machine; KNN: K-nearest neighbors; RF: Random forest; PNN: Probabilistic neural network; HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia; ICC: Intrahepatic cholangiocarcinoma; IPMN: Intra-ductal papillary mucinous neoplasm.

abdomen CT. Features were extracted from the segmented liver in all the phases, which were previously registered. Using 5-fold cross validation, they reported an accuracy of 86.9% and 81% for the detection of cirrhosis and HCC, respectively.

There are also several reports exploring the role of DL in the characterization of focal liver lesions (Figure 5). In this sense, Matake *et al*[52] applied an ANN to assist in the diagnosis of hepatic mases using clinical and radiological parameters extracted from CT images. The authors used 120 cases of liver diseases and implemented a leave-one-out cross-validation method for training and testing the ANN, reporting an AUC of 96.1%. Also using CT images, Yasaka *et al*[53] used a CNN for the differentiation of five different types of liver masses from contrast-enhanced CT. For testing, they used 100 liver mass images, reporting an accuracy of 84%. Similarly, Khan and Narejo[54] proposed Fuzzy Linguistic Constant (FLC) to enhance low contrast CT images of the liver before training a SVM to distinguish between cancerous or non-cancerous lesions. The reported classification accuracy was 98.3%. The proposed method also showed the ability to automatically segment the tumor with an improved detection rate of 78% and a precision value of 60%.



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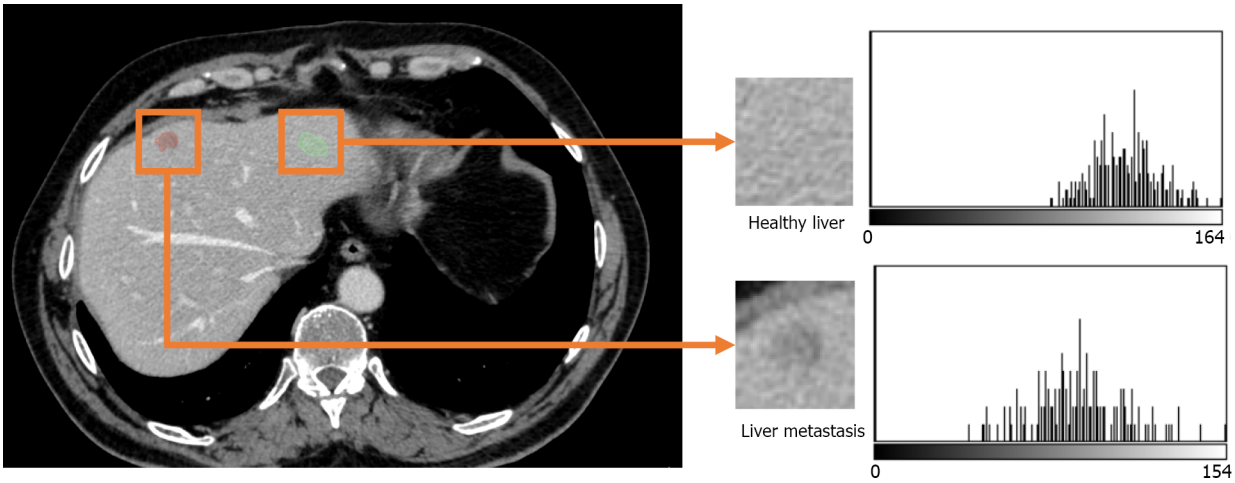
Figure 4 In-house experience on liver assessment with artificial intelligence. Magnetic resonance studies of a patient with liver focal lesions (liver hemangiomas), processed with the Liver Analysis research application from Siemens Healthcare. A: Automatic segmentation of the whole liver, liver segments, and other abdominal organs; B: Automatic detection, segmentation, and measurement of the two liver hemangiomas.

Liver and biliary system MRI

Techniques concerning MR images have also been developed for the diagnosis and classification of focal liver lesions (Figure 6). Zhou *et al*[55] proposed a method using a novel CNN to grade HCC from DWIs. They applied a 2D CNN to log maps generated from different b-value images. In their work, they reported a validation AUC of 83% using 40 cases. A CNN was also trained by Hamm *et al*[56] and Wang *et al*[57] to classify six different focal hepatic lesions from T1-weighted MR images in the postcontrast phase. They used 60 cases for testing and reported a sensitivity and specificity of 90% and 98%, respectively. In the second part of their study, they transformed it into an "interpretable" DL system by analyzing the relative contributions of specific imaging features to its predictions in order to shed light on the factors involved in the network's decision-making process. Finally, DCE-MRI and T2-weighted MRI, together with risk factor features, were applied to build an extremely randomized trees classifier for focal liver lesions[58], achieving an overall accuracy of 77%.

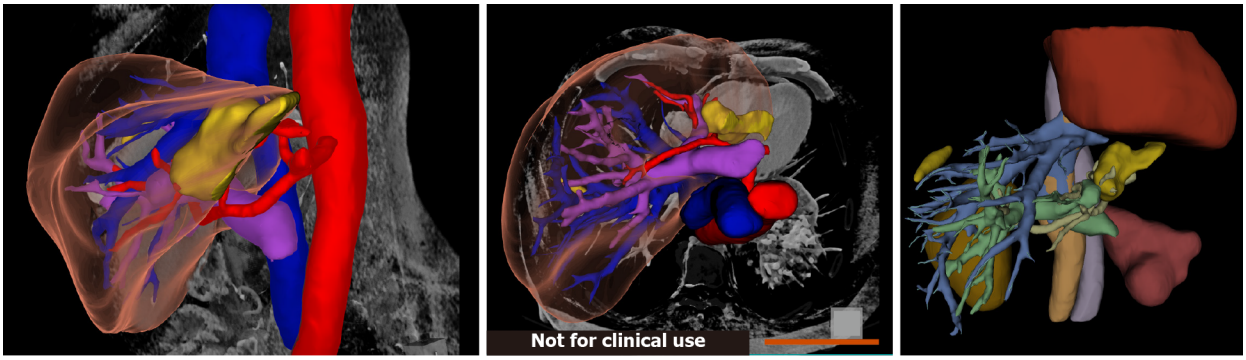
Some advancements have also been reached in the automatic diagnosis of lesions in the biliary system from MR cholangiopancreatography (MRCP) sequences. Logeswaran[59,60] trained an ANN classifier for assisting in the diagnosis of cholangiocarcinoma. He utilized 55 MRCP studies for testing and reported an accuracy of 94% when differentiating healthy and tumor images and of 88% in multi-disease tests.

MRI is a superior technique in the evaluation of chronic liver disease in comparison with CT, but making the most of it requires considerable skills and optimization at the acquisition, post-processing, and interpretation phases[61]. AI has proved useful to assist radiologists in the MR-guided diagnosis and grading of these diseases, including liver fibrosis and non-alcoholic fatty liver disease[62].



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Figure 5 Computerized tomography scan of a 61-year-old male patient with colon carcinoma and liver metastases. The intensity histograms of regions with and without metastases are different; hence, the first order radiomics features[109], which are based on the intensity histogram will potentially be different.



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Figure 6 Sixty-seven-year-old patient with pancreatic carcinoma and liver metastases treated with chemotherapy. The Digital Oncology Companion (Siemens Healthineers, Germany) artificial intelligence-based prototype automatically segments liver, portal and hepatic vessels, lesions, and surrounding anatomical structures. From left to right: screenshots of the segmented liver, vessels, and lesions; and generated 3D models.

Radiomics studies have been proposed to aid in the diagnosis of liver fibrosis. Kato *et al*[63] performed texture analysis of the liver parenchyma processed by an ANN to detect and grade hepatic fibrosis, with varying success depending on the type of MR sequence used (AUC of 0.801, 0.597, and 0.525 for gadolinium-enhanced equilibrium phase, T1-weighted, and T2-weighted images, respectively).

Later, Hectors *et al*[64] developed a DL algorithm for liver fibrosis staging using gadolinium enhancement sequences acquired in the hepatobiliary phase, which showed good to excellent diagnostic performance, comparable to that of MR elastography.

Liver-US

Ultrasound (US) and endoscopic ultrasonography (EUS) are commonly used in the diagnostic work-up of several pancreatic and liver lesions. AI-based solutions have also been applied to US images in the assessment of focal and diffuse liver diseases in order to enhance their diagnostic capabilities. Acharya *et al*[65] suggested a method for aiding in the diagnosis of focal liver lesions from liver US images. The authors extracted features from US images and trained several classifiers, obtaining the highest AUC (94.1%) using a probabilistic neural network (PNN) classifier. Another approach is shown in Yao *et al* [66], where a radiomics analysis was established for the diagnosis and clinical behavior prediction of HCC, showing an AUC of 94% for benign and malignant classification. Rightly, CNN architectures have also been developed for US images as in the report by Schmauch *et al*[67], where a CNN was employed to help in the diagnosis of focal liver lesions from US images. The authors used a dataset composed by 367 2D US images for training and another dataset from 177 patients for testing, reporting a mean score of 89.1%.

There is limited experience in the use of AI with US images with regards to diffuse liver disease. Li *et al*[68] used a SVM classifier to help in the diagnosis of fatty liver from US images. Input features were computed from ROIs selected by examiners. A total of 93 images were used for training and testing using leave-one-out cross-validation. The authors reported an 84% accuracy for normal livers and 97.1% for fatty livers. Moreover, a mix of radiomics features and DL techniques were used with two-dimensional shear waver elastography (2D-SWE) for assessing liver fibrosis stages in Wang *et al*[69]. Results reached AUCs of 97% for cirrhosis, 98% for advanced fibrosis, and 85% for significant fibrosis.

Pancreas CT and PET/CT

The role of AI in the detection of pancreatic lesions from CT has been extensively investigated. Pancreatic cancer detection is a challenging task for radiologists and its improvement is a hot research topic. Chen *et al*[70] developed a DL-based tool including a segmentation CNN and a 5-CNN classifier for the detection of pancreatic cancer lesions, with a special focus on lesions smaller than 2 cm, in abdominal CT scans. Their model was able to distinguish between cancer and control scans with an AUC of 0.95, 89.7% sensitivity, and 92.8% specificity. Sensitivity for the detection of lesions smaller than 2 cm was 74.7%[70]. Still focused on the identification of lesions smaller than 2 cm, Alves *et al*[71] proposed an automatic framework for pancreatic ductal adenocarcinoma (PDAC) detection based on state-of-the-art DL models. They trained an nnUnet (nnUnet_T) on a dataset including contrast-enhanced CT scans from 119 PDAC patients and 123 healthy individuals for automatic lesion detection and segmentation. Additionally, two other nnUnets were trained to investigate the impact of anatomy integration, with nnUnet_TP segmenting both the pancreas and the tumor and nnUnet_MS segmenting the pancreas, tumor, and adjacent anatomical structures. All three networks were compared on an open access external dataset, with nnUnet_MS offering the best results with an AUC of 0.91 for the entire dataset and of 0.88 for lesions smaller than 2 cm[71]. Several studies have focused on the role of AI-based solutions in the detection of pancreatic cystic lesions. Wei *et al*[72] presented a ML-based computer-aided diagnosis system to help in the diagnosis of pancreas serous cystic neoplasms from CT images. They extracted radiomic features from manual ROIs outlining the peripheral margin of each neoplasm. After selecting the most important features by using least absolute shrinkage selection operator regression, they trained a SVM classifier by a 5-fold cross validation with 200 patients. The authors used a validation cohort of 60 patients and reported and AUC of 83.7%, a sensitivity of 66.7%, and a specificity of 81.8%. Along the same lines, Li *et al*[73] also proposed a computer-aided framework for early differential diagnosis of pancreatic cysts without pre-segmenting the lesions by using densely connected convolutional networks (Dense-Net). In this approach, saliency maps were integrated in the framework to assist physicians to understand the decisions of the DL methods. Accuracy reported on a cohort of 206 patients with four pathologically confirmed subtypes of pancreatic cysts was 72.8%, significantly higher than the baseline of 48.1% according to the authors. Park *et al*[74] developed a 3D nnU-Net-based model for the automatic diagnosis of solid and cystic pancreatic neoplasms on abdominal CT scans. The model was trained on CT scans (852 patients) from both patients who underwent resection for pancreatic lesions and subjects without any pancreatic abnormalities, and performance was evaluated using receiver operating characteristic analysis in a temporally independent cohort (test set 1, including 603 patients) and a temporally and spatially independent cohort (test set 2, including 589 patients). This approach showed a remarkable capacity to identify solid and cystic pancreatic lesions on CT, with an AUC of 0.91 for the test set 1 and 0.87 for the test set 2. Furthermore, it offered a high sensitivity in the identification of solid lesions of any size (98%-100%) and cystic lesions of at least 1 cm (92%-93%)[74].

In the pursuit of more accurate models, some authors have combined CT images with other biomarkers, such as molecular markers or multimodal images. For example, Qiao *et al*[75] used CT scans and serum tumor markers (including serum carbohydrate antigens 50, 199, and 242) to train different types of networks (CNN, FCN, and U-Net) to diagnose pancreatic cancer with high sensitivity and specificity. Li *et al*[76] also used a hybrid SVM-RF model to classify normal and pancreas cancer from PET/CT images. First, they segmented the pancreas from CT images and registered the CT and PET series, then they extracted features from the segmented ROI in both types of studies. The authors tested the model using 10-fold cross validation with 80 cases and achieved 96.47% accuracy, 95.23% sensitivity, and 97.51% specificity.

Pancreas-MRI

MR is the technique of election for the assessment of complex pancreatic conditions. Thus, its association with AI is regarded as promising to help radiologists in diagnostic dilemmas regarding this organ. For instance, radiomics has been proposed as a way to predict the malignant potential of pancreatic cystic lesions, differentiating benign cysts from those likely to transform into pancreatic cancer[77].

There is limited experience with the use of AI in the detection of focal lesions with pancreatic MR studies. Corral *et al*[78] proposed the use of SVM to classify intraductal papillary mucinous neoplasms (IPMN). First, features were extracted using a CNN from T2-weighted and post-contrast T1-weighted MR images. For validation, authors used 10-fold cross-validation using 139 cases. They achieved an AUC of 78%. Kaissis *et al*[79] also developed a supervised ML algorithm which predicted the above-

versus-below median overall survival of patients with pancreatic ductal adenocarcinoma, with 87% sensitivity and 80% specificity, using preoperative DWIs.

Lastly, the generation of synthetic MR images of pancreatic neuroendocrine tumors (PNET) has been explored using GANs. This data augmentation technique can alleviate the relative low abundance of these type of pancreatic tumors in order to train AI models. Gao and Wang then used the synthetic images to evaluate the performance of a CNN in the prediction of PNET grading on contrast-enhanced images[80].

Pancreas-EUS

Application of AI to EUS has focused on the differentiation of focal pancreatic lesions. In this sense, Săftoiu *et al*[81] developed an ANN to help in the difficult differentiation between PDAC and focal chronic pancreatitis (CP) with EUS-elastography. They included 258 patients in the study and reported 84.27% testing accuracy using 10-fold cross-validation. In addition, Kuwahara *et al*[82] used a CNN to assist in the distinction between benign and malignant IPMNs of the pancreas from EUS images. For testing, the authors used images from 50 patients, obtaining an AUC of 98% and sensitivity, specificity, and accuracy values of 95.7%, 92.6%, and 94%, respectively. Finally, in the report by Marya *et al*[83] an EUS-based CNN model was trained to differentiate autoimmune pancreatitis (AIP) from PDAC, CP, and normal pancreas (NP). Results obtained from 583 patients (146 AIP, 292 PDAC, 72 CP, and 73 NP) demonstrated a sensitivity of 99% and a specificity of 98% to distinguish between AIP and NP, 94% and 71% for AIP and CP, and 90% and 93% for AIP and PDAC. Furthermore, the sensitivity and specificity to distinguish AIP from all study conditions (*i.e.*, PDAC, CP, and NP) were 90% and 85%, respectively. In view of these results, the application of AI to EUS in the assessment of focal pancreatic lesions is promising, although limited due to the short number of available databases for algorithm training and validation[84].

TREATMENT PREDICTION

Prediction of treatment response and patient outcome based on AI is a very appealing idea which has been explored in a number of liver and pancreatic diseases, particularly in patients with HCC (Table 3).

The idea of using ML to predict the prognosis of patients with HCC emerged decades ago. Already in 1995 the progression of hepatectomized patients with HCC was analyzed using ANN[85]. Liver volume, which was measured in CT studies, was used, among others, as an input parameter. Fifty-four example cases were used to train an ANN composed of three layers, and the model was successfully used to predict the prognosis of 11 patients. Nevertheless, the model was not tested with enough cases to determine its usefulness in actual clinical activity. However, the rise of AI has prompted many more works to be developed in the last few years. The response to intra-arterial treatment of HCC prior to intervention has been predicted using ML[86,87]. Specifically, logistic regression (LR) and RF models were trained with 35 patients using features extracted from clinical data and the segmentations of liver and liver lesions in a contrast-enhanced 3D fat-suppressed spoiled gradient-echo T1-weighted sequence in the arterial phase. Both trained models predicted treatment response with an overall accuracy of 78% (62.5% sensitivity, 82.1% specificity). Other authors tried to predict the early recurrence of HCC employing a CNN model based on the combination of CT images and clinical data[88]. They used 10-fold cross-validation with data from 167 patients and reported an AUC of 0.825. A RestNet CNN model was also trained for preoperative response prediction of patients with intermediate-stage HCC undergoing transarterial chemoembolization[89]. The model used the segmented ROI of the tumor area in a CT study as input. The training cohort included 162 patients and the two validation cohorts included 89 and 138 patients, respectively. The authors reported an accuracy of 85.1% and 82.8% in the two evaluation datasets.

Radiomics has also been applied to predict treatment response of HCC to different therapies based on studies of several imaging modalities. The early recurrence of HCC after curative treatment was evaluated using an LR model based on radiomics features[90], which were extracted from manually delineated peritumoral areas in CT images. They used 109 patients for training and 47 patients for validation, reporting an AUC of 0.79 with the validation dataset. Guo *et al*[91] also predicted the recurrence of HCC after liver transplantation. For that purpose, authors extracted radiomic features from ROIs delineated around the lesion in arterial-phase CT images. Then, they combined clinical risk factors and radiomic features to build a multivariable Cox regression model. The authors used a training dataset of 93 patients and a validation dataset of 40 patients and they reported a C-index of 0.789 in the validation dataset.

ML models have also been used to predict hepatobiliary toxicity after liver SBRT[92]. The authors built a CNN model which was previously pretrained using CT images of human organs. Then, using transfer learning, the model was trained with liver SBRT cases. They used 125 patients for training and validation using a 20-fold cross-validation approach, reporting an AUC of 0.79.

Regarding the pancreas, postoperative pancreatic fistulas were predicted using ML-based texture analysis[93] performed to extract features from ROIs segmented in non-contrast CT images. Then, after

Table 3 Summary of the works proposed to predict patient prognosis using artificial intelligence

Anatomical area	Pathology	Modality	AI model	What is prognosed?	Ref.
Liver	HCC	CT	ANN	Progression of hepatectomized patients with HCC	[85]
			CNN	Early recurrence of HCC	[88]
				Response to transarterial chemoembolization for patients with intermediate-stage HCC	[89]
			LASSO Cox regression	Early recurrence of HCC	[90]
				Recurrence of HCC after liver transplantation	[91]
				Recurrence of HCC after resection	[118]
		MRI	LR, RF	Response to intra-arterial treatment of HCC	[86,87]
		US	CNN, SVM	Response to transarterial chemoembolization for patients with HCC	[119]
Biliary system	Liver metastases, HCC, cholangiocarcinoma	CT	CNN	Prediction of hepatobiliary toxicity after liver SBRT	[92]
Pancreas	Postoperative pancreatic fistula	CT	RepTree	Prediction of postoperative pancreas fistulas after pancreatoduodenectomy	[93]

HCC: Hepatocellular carcinoma; CT: Computerized tomography; MRI: Magnetic resonance imaging; US: Ultrasound; ANN: Artificial neural network; CNN: Convolutional neural network; LR: Logistic regression; RF: Random forest; SVM: Support vector machine; SBRT: Stereotactic body radiotherapy.

dimension reduction, several ML classifiers were built using Auto-WEKA 2.0, obtaining the best results using a REPTree classifier. The authors used 10-fold cross-validation using data from 110 patients, and reported an AUC of 0.95, sensitivity of 96%, and specificity of 98%.

DISCUSSION

In recent years, a large number of AI-based solutions have been developed with the aim of easing and streamlining the radiologist's workflow. Many of these tools are focused on imaging of the liver, biliary system, and pancreas. The developed tools range from improving image quality to the prediction of the patient's prognosis after treatment. The literature shows that many AI-based solutions targeting liver and pancreas imaging allow for improved disease detection and characterization, lower inter-reader variability, and increased diagnostic efficiency. A key factor for their success in the clinical setting is to attain a seamless integration in the radiologist's workflow, requiring minimal additional work by the radiologist and adding significant value to the radiologist's work. In this sense, it is crucial that there is a fluid collaboration between the radiologists, technicians, and bioengineers in charge of the tools.

Image analysis and processing are transversal parts of most AI methods described in this review. Improving their performance is thus a key task. Unfortunately, some image processing techniques such as registration are still time-consuming, hence making the incorporation of some of these procedures in clinical practice unfeasible. Some new methods are arising to minimize this impact[94], especially in critical applications like image IGS. Semi-automatic or even automatic segmentation is another important step that some of the AI tools may incorporate for diagnosis or prognosis purposes[95]. Therefore, it is of paramount importance for these algorithms to achieve a high level of performance.

The literature reports many applications of AI to aid in the detection and characterization of pancreatic and liver focal lesions using a variety of imaging modalities as input, either single (*e.g.*, T1-weighted MRI) or in combination with other techniques and data (*e.g.*, T2-weighted and DCE-MRI plus risk factors). In chronic liver disease, radiomics-based tools have been developed to assist in the diagnosis and grading of hepatic fibrosis, among others. These models have been built using different imaging modalities, such as MRI or US.

With regard to the prognosis of liver, biliary or pancreatic diseases, tools based on radiological information have hardly been developed. Many of these tools are focused on the prognosis of HCC based on information extracted from CT[96]. In this field of research, literature shows a clear trend toward integrating genetic information[97-101]. There are also studies that try to include variables extracted from clinical data and laboratory values[102,103]. In a scenario that advances towards integrated diagnosis, increasing volumes of data of different nature are available. This should allow for the generation of more accurate predictive models of clinical prognosis using information from many sources.

For the AI-based tools developed to be used in daily clinical practice, they must obtain regulatory clearance, such as Food and Drug Administration (FDA) approval in the United States or CE marking in Europe. Despite the explosive production of such tools in the last years, to date only a small group of them have obtained this approval. One of the main problems is the lack of appropriately annotated data. Without large datasets of properly labeled studies, the performance of data-hungry algorithms like CNNs will not be sufficient to be massively deployed in clinical environments. Furthermore, algorithms demand diverse data, such as multi-centric and multi-vendor, to avoid selection biases that would challenge their implementation in a real-world environment[104]. Another limitation of most AI-based tools found today is that they are aimed at a very concrete application (narrow AI applications), within a specific imaging modality, rather than being valid for a wide range of tasks at the radiologist's work practice.

Yet, the general attitude of radiology staff toward AI is positive. In a recent survey, European radiographers declared excitement about AI (83%), although only 8% had been taught on this matter in their qualification studies[105].

In another survey, European radiologists regarded the outcomes of AI algorithms for diagnostic purposes as generally reliable (75.7%), and algorithms for workload prioritization as very helpful (23.4%) or moderately helpful (62.2%) to reduce the workload of the medical staff[106].

The sentiment of gastroenterologists toward AI is also generally favorable, with a wide majority of United Kingdom[107] and European[108] specialists perceiving it as beneficial to key aspects of their clinical practice. Their main concerns according to these studies are related to algorithm bias, lack of guidelines, and potential increase in procedural times and operator dependence.

CONCLUSION

The rapid advance of AI is already transforming the gastrointestinal field with the development of applications aimed to assist and streamline image diagnosis. Traditional diagnostic imaging techniques such as US, EUS, CT, MRI, and PET/CT are already benefitting from a variety of AI algorithms that can perform automatic or semi-automatic segmentation and registration of the liver and pancreas and their lesions, aid the diagnosis and characterization of pancreatic and liver focal lesions and diffuse illnesses, improve image quality, accelerate image acquisition, and anticipate treatment response and patient prognosis. Moreover, with the use of radiomics, AI can add quantitative information previously undetected by radiologists to radiological reports. The massive adoption of AI in radiology of pancreatic and liver diseases is still incipient, but irreversible, and the sector is clearly moving in this direction. Advances in the field, such as the availability of regulatory cleared, robust algorithms trained and validated multicentrically, increased awareness on AI by the medical staff, and access to products that seamlessly integrate with their workflow should pave the way for a rapid adoption of AI in the clinical practice, impacting the outcomes of hepatic and pancreatic patients for the better.

FOOTNOTES

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MINIREVIEWS

Role of noncoding RNAs in liver fibrosis

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Abstract

Liver fibrosis is a wound-healing response following chronic liver injury caused by hepatitis virus infection, obesity, or excessive alcohol. It is a dynamic and reversible process characterized by the activation of hepatic stellate cells and excess accumulation of extracellular matrix. Advanced fibrosis could lead to cirrhosis and even liver cancer, which has become a significant health burden worldwide. Many studies have revealed that noncoding RNAs (ncRNAs), including microRNAs, long noncoding RNAs and circular RNAs, are involved in the pathogenesis and development of liver fibrosis by regulating signaling pathways including transforming growth factor-β pathway, phosphatidylinositol 3-kinase/protein kinase B pathway, and Wnt/β-catenin pathway. NcRNAs in serum or exosomes have been reported to tentatively applied in the diagnosis and staging of liver fibrosis and combined with elastography to improve the accuracy of diagnosis. NcRNAs mimics, ncRNAs in mesenchymal stem cell-derived exosomes, and lipid nanoparticles-encapsulated ncRNAs have become promising therapeutic approaches for the treatment of liver fibrosis. In this review, we update the latest knowledge on ncRNAs in the pathogenesis and progression of liver fibrosis, and discuss the potentials and challenges to use these ncRNAs for diagnosis, staging and treatment of liver fibrosis. All these will help us to develop a comprehensive understanding of the role of ncRNAs in liver fibrosis.

Key Words: MicroRNAs; Long noncoding RNAs; Circular RNAs; Liver fibrosis;

Diagnosis; Treatment

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Core Tip: Liver fibrosis is an inevitable stage in the development of various chronic liver diseases, and manifests as an imbalance between the formation and degradation of extracellular matrix. The key mechanism of liver fibrosis is the activation of hepatic stellate cells, which is coordinately regulated by a variety of cytokines, inflammatory factors and chemokines involved in multiple cells signaling pathways. In this review, we discuss the role of noncoding RNAs (ncRNAs) in regulating the signaling pathways in the formation and regression of liver fibrosis, and the limitations, challenges, and prospects of ncRNAs in the diagnosis and treatment of liver fibrosis.

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INTRODUCTION

Liver fibrosis is the result of excessive accumulation of extracellular matrix (ECM) caused by continuous liver injuries that promote wound healing[1]. Liver injuries can be caused by many factors including persistent hepatitis B virus (HBV)/hepatitis C virus (HCV) infections, excessive alcohol consumption, metabolic diseases, drugs, genetic diseases, cholestasis, and autoimmune diseases. Due to an increase in the prevalence of obesity and type 2 diabetes, liver fibrosis caused by nonalcoholic steatohepatitis (NASH) has been increasing annually in recent years[2]. Liver fibrosis can resolve at an early stage if the injuries subside. Progressive fibrosis is associated with architectural changes to hepatic lobules and may lead to cirrhosis, liver failure, portal hypertension, and even hepatocellular carcinoma (HCC).

Hepatic stellate cells (HSCs) play a central role in liver fibrosis. HSCs, also known as perisinusoidal cells, are located in the Disse space under healthy conditions. When injury occurs, HSCs are activated and transdifferentiate into myofibroblast-like cells which are the main source of ECM[3]. Hepatic fibrosis is a dynamic process coordinated by multiple cells in the liver. Acute injury, such as viral infection, induces an inflammatory response, necrosis, and apoptosis in hepatocytes which leads to liver regeneration and limited ECM deposition. However, if the damage persists, the injured hepatocytes attract an infiltration of inflammatory cells such as T lymphocytes and neutrophils, which will in turn activate HSCs by releasing cytokines, chemokines, and reactive oxygen species (ROS). Activated HSCs can maintain the active state by the mediators produced by the autocrine and paracrine system. In addition, platelet-derived growth factor (PDGF) secreted by liver macrophages (Kupffer cells) stimulates the continuous proliferation of HSCs. Therefore, inhibiting the activation and proliferation of HSCs, promoting the apoptosis of activated HSCs, and reducing the expression of fibrogenic factors are considered to be the key measures for the successful treatment of liver fibrosis.

Noncoding RNAs (ncRNAs) refer to RNAs that are transcribed from the genome but do not normally encode proteins, although some of them have recently been reported to encode small proteins[4]. According to their length, ncRNAs can be divided into short ncRNAs and long ncRNAs (lncRNAs). MicroRNAs (miRNAs) are a class of short ncRNAs of approximately 22 nucleotides in length that act as gene repressors by complementary binding to the 3' untranslated region of target mRNA to degrade or prevent it from being translated to protein[5,6]. LncRNAs are defined as ncRNAs longer than 200 bp with 5′-end m7G caps and 3′-end poly(A) tails. LncRNAs can regulate gene expression in *cis* or *trans*, change the structure and function of chromatin *via* interaction with proteins, or act as competitive endogenous RNAs (ce-RNAs) for post-transcriptional regulation[7]. Circular RNAs (circRNAs) are a novel form of ncRNAs with a covalently closed single-stranded structure, which is formed by back-splicing of the 3' and 5' ends of mRNAs[8]. Depending on their subcellular localization, circRNAs have different biological functions: interfering with signal transduction pathways and regulating the transcription and translation of target genes, sponge proteins and miRNAs[9].

In this review, we summarize the latest findings about miRNAs, lncRNAs, and circRNAs in the pathogenesis and progression of liver fibrosis and discuss the potential of ncRNAs as biomarkers for diagnosis and as therapeutic targets for liver fibrosis.

NCRNAS IN THE PATHOGENESIS AND PROGRESSION OF LIVER FIBROSIS

The activation and proliferation of HSCs are essential steps in the development of liver fibrosis. Numerous studies have shown that ncRNAs exert profibrotic effects by regulating genes in the activation and proliferation signaling pathways of HSCs. Signaling pathways closely related to liver fibrosis mainly include transforming growth factor-β (TGF-β)/Smad, phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase 1 (AKT), Wnt/β-catenin, and nuclear factor κ light chain enhancer of activated B cells (NF-κB) pathways. Although some miRNAs[10] and lncRNAs[11] involved in liver fibrosis have been reviewed elsewhere, we focus on the most recent data published in the past 3 years as summarized in Figure 1.

ncRNAs regulating TGF-β/Smad signaling pathway in liver fibrosis

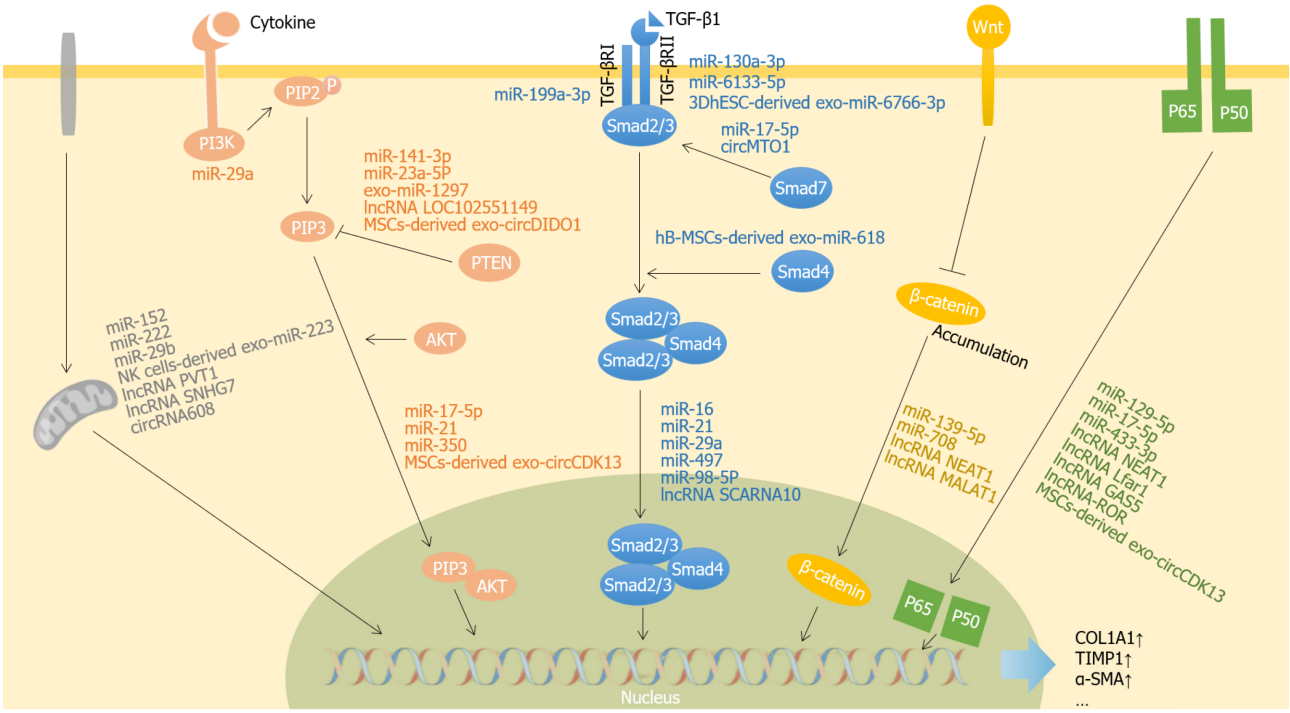
Transforming growth factor-β1 (TGF-β1) is a well-recognized fibrogenic cytokine that is widely expressed in damaged hepatocytes, Kupffer cells, HSCs, sinusoidal endothelial cells, and platelets. TGF-β1 promotes HSC activation through a canonical (Smad) or noncanonical pathway[12]. In the TGF-β1/Smad signaling pathway, TGF-β1 binds to the TGF-β type II receptor (TGF-βRII) on the cell membrane and then recruits the TGF-β type I receptor (TGF-βRI) to form a heterotetrameric complex. This complex induces the phosphorylation of intracellular Smad2 and Smad3, which then bind with Smad4 and translocate to the nucleus to regulate expression of target genes[13-15]. In addition, TGF-β1 induces the expression of Smad7, which maintains the balance between profibrotic and antifibrosis by negatively regulating TGF-βRI and Smad2[13].

Many miRNAs are involved in the regulation of the TGF-β/Smad signaling pathway and liver fibrosis. These miRNAs include miR-21, miR-497, miR-16, miR-98-5p, miR-199a-3p, miR-29a, and miR-130a-3p. MiR-21 is expressed abundantly in liver, is present in serum, and is positively associated with liver inflammation, fibrosis, and cancer[16]. TGF-β1 induces transcription, processing and maturation of pri-miR-21 through a Smad3-dependent pathway, while mature miR-21 promotes the development of fibrosis by targeting the inhibitory Smad gene-small mothers against decapentaplegic7[15]. *Clonorchis sinensis* promotes hepatic fibrosis by inducing miR-497 and activating the TGF-β/Smad pathway[17]. Pan *et al*[18] revealed that miR-16 plays an essential role in the phenotypic remodeling of myofibroblasts. Overexpression of miR-16 restored the phenotype of HSCs and led to fibrotic regression by targeting Smad2 and Wnt3a to interfere with TGF-β and Wnt signaling pathways, respectively[18]. In patients with chronic HBV-induced liver fibrosis, expression of miR-98-5p was significantly downregulated. Further studies indicated that overexpression of miR-98-5p significantly inhibited HSC activation through targeting the TGF-β1/Smad3 signaling pathway[19]. Yang *et al*[20] demonstrated that expression of miR-199a-3p was upregulated in carbon tetrachloride (CCl_4)-induced liver fibrotic rats, and miR-199a-3p activated HSCs by targeting caveolin-2 (CAV2) to increase expression of TGF-βRI. Stimulation with TGF-β resulted in the downregulation of miR-29a, which increased follistatin-like 1 expression and accelerated the progression of fibrosis by enhanced phosphorylation of Smad2[21]. MiR-130a-3p was significantly decreased in liver fibrosis caused by *Schistosoma japonicum*[22]. It has been shown that miR-130a-3p attenuates fibrosis by inhibiting the activation and proliferation of HSCs and promoting their apoptosis through regulation of mitogen-activated protein kinase 1 and TGF-βRI/II both *in vitro* and *in vivo*[22].

In addition to miRNAs, lncRNAs and circRNAs are associated with the TGF-β pathway and liver fibrogenesis. LncRNA small Cajal body-specific RNA 10 (lncRNA SCARNA10) was found to inhibit the expression of polycomb repressive complex 2 to induce hepatocytes apoptosis and HSC activation, thereby stimulating the TGF-β pathway and liver fibrogenesis[23]. CircRNA mitochondrial tRNA translation optimization 1 (circMTO1) was reported to inhibit liver fibrosis through interaction with miR-17-5p and Smad7[24].

ncRNAs regulating PI3K/AKT signaling pathway in liver fibrosis

The PI3K/AKT pathway is an essential intracellular signaling pathway in the regulation of the cell cycle. The AKT cascade can be activated by cytokine receptors such as receptors of TGF-β and PDGF. PI3K is activated to induce phosphorylation of Phosphatidylinositol-4,5-biophosphate (PIP2) on the cell surface, leading to production of phosphatidylinositol-3,4,5-trisphosphate (PIP3). AKT (also known as protein kinase B, PKB) binds to PIP3 and they are co-translocated to the nucleus, where they regulate target gene expression to stimulate cell proliferation and inhibit apoptosis. Phosphatase and tensin homology deleted on chromosome ten (PTEN) increases the number of activated HSCs by catalyzing dephosphorylation of PIP3 and downregulating the PI3K/AKT signaling pathway. A variety of miRNAs regulate the PI3K/AKT signaling pathway. MiR-21 is significantly upregulated in liver fibrosis induced by cadmium exposure, which leads to the progression of fibrosis by activating the PI3K/AKT pathway[25]. Lipotoxic hepatocyte-derived exosomal miR-1297 promotes HSC proliferation and activation by inhibiting expression of PTEN[26]. MiR-23a-5p activates the PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathway by inhibiting PTEN and can be targeted by lncRNA LOC102551149 to reduce liver fibrosis[27]. All these results indicate that ncRNAs, especially miRNAs and lncRNAs, play important roles in liver fibrosis through targeting the PI3K/AKT pathway.



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Figure 1 Reported pathways and targets of noncoding RNAs involved in liver fibrosis. Noncoding RNAs regulate the target gene transcription in the pathogenesis and progression of liver fibrosis through inhibiting or activating the key genes in different signaling pathways. PI3K: Phosphatidylinositol 3-kinase; AKT: Serine/threonine kinase 1; PIP2: Phosphatidylinositol-4,5-biophosphate; PTEN: Phosphatase and tensin homology deleted on chromosome ten; Col1A1: Collagen 1A1; TIMP1: Targeted tissue inhibitor of metalloproteinase1; α-SMA: α-smooth muscle actin; PIP3: Phosphatidylinositol-3,4,5-trisphosphate; TGF-β: Transforming growth factor-β; TGF-βRII: TGF-β type II receptor; MSC: Mesenchymal stem cell; hB-MSC: Human bone MSC; hT-MSC: Human tonsil-derived MSC; 3DhESC: 3D-cultured human embryonic stem cells;PVT1: Plasmacytoma variant translocation 1; SNHG7: Small nucleolar RNA host gene 7; DIDO1: Death inducer-obliterator 1; CDK13: Cyclin dependent kinase 13; MTO1: Mitochondrial tRNA translation optimization 1; SCARNA10: Small Cajal body-specific RNA 10; MALAT1: Metastasis-associated lung adenocarcinoma transcript1; NEAT1: Nuclear enriched abundant transcript1; Lfar1: Liver fibrosis associated lncRNA1; GAS5: Growth arrest-special transcript 5; ROR: Regulator of reprogramming.

ncRNAs regulating Wnt/β-catenin signaling pathway in liver fibrosis

Wnt/β-catenin is involved in the development of fibrosis of several tissues, including kidney, lung, skin, and liver. Wnt proteins are cysteine-rich glycoproteins generally secreted to the ECM. β-Catenin is a cytoplasmic protein that can be activated by Wnt and is translocated to the nucleus to activate transcription of target genes, thereby regulating occurrence of fibrosis[28]. Yang *et al*[29] demonstrated that expression of miR-708 was downregulated in fibrotic liver tissue. The authors further demonstrated that overexpression of miR-708 inhibited activation of HSCs by targeting zinc finger E-box binding homeobox 1 and regulating the Wnt/β-catenin signaling pathway[29]. Different forms of ncRNAs may work together to have a synergistic effect in the pathogenesis and progression of liver fibrosis. For example, lncRNA nuclear enriched abundant transcript1 (lncRNA NEAT1) and miR-139-5p have a synergistic effect that exacerbates the development of liver fibrosis[30]. Another study has revealed that lncRNA metastasis-associated lung adenocarcinoma transcript1 (lncRNA MALAT1) upregulates expression of β-catenin and promotes liver fibrosis through the Wnt/β-catenin pathway[31].

ncRNAs regulating NF-κB signaling pathway in liver fibrosis

NF-κB is one of the transcription factors that regulates important cellular events, particularly inflammation. NF-κB consists of two subunits p50 and p65, which can be activated by extracellular signals. Activated NF-κB translocate to the nucleus to regulate expression of various cytokines, growth factors, and other target genes. LncRNA NEAT1 plays critical roles in hepatic fibrosis of different etiologies by targeting various miRNAs associated with NF-κB signaling pathways. In NASH-induced liver fibrotic mice, Zhang *et al*[32] found that lncRNA NEAT1 stimulated expression of paternally expressed gene 3 (PEG3) by inhibiting miR-129-5p, which reduced HSC apoptosis through the NF-κB (p65/p50) signaling pathway. The effect of the lncRNA NEAT1/miR-129-5p axis on liver fibrosis had also been confirmed in alcoholic steatohepatitis mice by targeting suppressor of cytokine signaling 2[33]. In addition, lncRNA NEAT1 also promotes fibrosis *via* inhibition of miR-148a-3p and miR-22-3p and regulation of cytohesin 3 expression[34]. LncRNA liver fibrosis associated lncRNA1 (lncRNA Lfar1) was demonstrated to promote hepatic fibrosis through activation of HSCs, probably by way of its regulatory effect on macrophages through the NF-κB signaling pathway[35]. Overexpression of lncRNA growth arrest-

special transcript 5 (lncRNA GAS5) decreased expression of miR-433-3p, which then intercepted the NF-κB signaling pathway through targeting of toll-like receptor 10[36]. In addition, lncRNA maternally expressed gene 3 (lncRNA MEG3) targeted NLR Family CARD Domain Containing 5 (NLRC5) to reverse liver fibrosis[37]. All of these results indicate that ncRNAs regulating liver fibrosis through targeting the NF-κB pathway are mainly lncRNAs, including lncRNAs NEAT1, Lfar1, GAS5, and MEG3.

ncRNAs regulating autophagy pathway in liver fibrosis

Autophagy is a process that regulates self-metabolism and maintains cellular homeostasis by removing cell debris, misfolded proteins and lipid droplets[38]. Activation of autophagy promotes liver fibrosis by increasing the digestion of lipid droplets and activating multiple signaling pathways, which implies that promoting regeneration of lipid droplets and restraining expression of proinflammatory factors inhibits liver fibrosis[38]. In hypoxic conditions, lncRNA plasmacytoma variant translocation 1 (lncRNA PVT1) regulates expression levels of autophagy-related gene (ATG)14 by decreasing miR-152, thereby activating HSCs through the autophagy pathway[39]. LncRNA small nucleolar RNA host gene 7 (lncRNA SNHG7) increased DNA methyltransferase 3 alpha (DNMT3A) expression through binding to miR-29b, which is involved in liver fibrosis and autophagy. Inhibition of lncRNA SNHG7 significantly decreases expression of collagen and autophagy factors, leading to inhibition of liver fibrosis[40]. In addition to lncRNAs, circRNAs are also associated with autophagy and mitophagy. Xu *et al*[41] illustrated that circRNA608/miR-222 regulates PTEN-induced putative kinase 1-mediated mitophagy and liver fibrosis in NASH-induced fibrotic mice.

Other ncRNAs targeting host genes involved in liver fibrosis

Chen *et al*[42] demonstrated that miR-451 and miR-185 were downregulated in activated HSCs, and they exerted antifibrotic effects synergistically by targeting erythropoietin-producing hepatocellular receptor B2. MiR-451 upregulated expression of miR-185. This occurs at the post-transcriptional level by targeting nuclear export receptor exportin 1 (XPO-1). Zhao *et al*[43] demonstrated that lncRNA molecule interacting with CasL2 (lncRNA Mical2) upregulated p66 Src homologous-collagen homologue (p66Shc) through sponging miR-203a-3p, which promoted reactive oxygen species (ROS)-mediated epithelial–mesenchymal transition and liver fibrosis. It has been reported that lncRNA X-inactive-specific transcript (lncRNA XIST) damages mitochondrial function and increases ROS production to promote HSC activation by regulating miR-539-3p and ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5)[44]. Studies from cholestatic liver injury caused by biliary atresia have indicated that expression of lncRNA H19 is significantly upregulated in exosomes derived from liver and serum. LncRNA H19 deficiency protects mice from liver fibrosis by inhibiting sphingosine-1-phosphate receptor 2/sphingosine kinase 2 activation and by sponging let-7 to upregulate high-mobility group AT-hook 2 expression[45]. It has also been reported that depletion of macrophages significantly reduced lncRNA H19 and inhibited cholestatic liver injury in bile duct ligation mice[46]. LncRNA actin alpha 2-antisense RNA 1 (lncRNA ACTA2-AS1) accelerated liver fibrosis and epigenetic activation by targeting the p300/ETS transcription factor (ELK1) complex in biliary diseases[47]. CircRNA F-box and WD repeat domain containing 4 (circFBXW4) was downregulated significantly in HSCs of mice with liver fibrosis. Overexpression of circFBXW4 inhibited HSC activation by targeting miR-18b-3p to increase FBXW7 expression[48]. Similarly, CircRNA CREB binding protein (circCREBBP) inhibited liver fibrosis by targeting miR-1291 to regulate the expression of left-right determinant cluster 2 (LEFTY2) [49]. Hsa_circ_0071410 inhibited activation of HSCs by binding to miR-9-5p in irradiation-induce liver fibrosis[50]. All these ncRNAs in the pathogenesis and progression of liver fibrosis are summarized in Table 1[51-60].

POTENTIAL APPLICATION OF NCRNAS IN THE DIAGNOSIS OF LIVER FIBROSIS

Liver-related mortality increases with the progression of fibrosis. Therefore, it is essential for the early diagnosis of liver fibrosis. At present, the gold standard for the diagnosis of liver fibrosis is still liver biopsy, although it has some limitations such as sampling error, inter- and intra-observer variability [61], invasiveness to patients, and many other complications. Several noninvasive examinations have been introduced in clinical settings, including serum markers, combined indices or scores, and imaging techniques. Hepascore and enhanced liver fibrosis score are based on serum liver fibrosis markers such as tissue metalloproteinases and hyaluronic acid [62]. Elastography, including ultrasound elastography and magnetic resonance elastography, is a method to access liver stiffness quantitatively and it is more accurate than serological markers for diagnosis of advanced liver fibrosis. However, elastography has disadvantages such as unreliable results due to high body mass index (BMI) and high cost, making it unsuitable for population screening[62]. As ncRNAs in the blood are easily accessible for detection, they have potential as novel noninvasive biomarkers for diagnosis of liver fibrosis.

Recent research has shown that stimulation of HSCs with TGF-β and PDGF-BB decreased the intracellular miR-29 expression level but significantly increased miR-29 level in the supernatant vesicles [63]. They verified the results in serum from patients with HCV-related liver fibrosis and mice with CCl_4

Table 1 Noncoding RNAs in the pathogenesis and progression of liver fibrosis

ncRNAs	Target genes	Signaling pathways	Ref.
miR-199a-3p	*CAV2*	TGF-β/Smad	[20]
miR-497	*Smad7*	TGF-β/Smad	[17]
miR-21	*Smad2/3/7*	TGF-β/Smad	[15]
	TGF-β	PI3K/AKT	[25]
	-	PPARα	[51]
	-	PDCD4/AP-1	[51,52]
	-	Smad7/Smad2/3/NOX4, Spry1/ERK/NF-κB	[51,53]
	-	HIF-1α/VEGF	[54]
miR-16	*Smad2*, *Wnt3a*	TGF-β/Smad, Wnt	[18]
miR-130a-3p	*TGF-βRI*, *TGF-βRII*; *MAPK1*	TGF-β; MAPK	[22]
miR-98-5p	*TGF-βRI*	TGF-β1/Smad3	[19]
miR-6133-5p	*TGF-βRII*, *FGFRI*	TGF-β/Smad2/3, AKT/ERK/JNK	[55]
miR-708	*ZEB1*	Wnt/β-catenin	[29]
exo-miR-1297	*PTEN*	PI3K/AKT	[26]
miR-350	*SPRY2*	PI3K/AKT and ERK	[56]
miR-34c	*ACSL1*	-	[57]
miR-200c	*HAS2*	-	[58]
miR-451, miR-185	*EphB2*	-	[42]
miR-20b-5p	*STAT3*	STAT3	[97]
lncRNA SNHG7	*miR-29b*, *DNMT3A*	Autophagy pathway	[40]
lncRNA PVT1	*miR-152*, *ATG14*	Autophagy pathway	[39]
lncRNA SCARNA10	*PRC2*	TGF-β	[23]
lncRNA LOC102551149	*miR-23a-5p*, *PTEN*	PI3K/AKT/mTOR/Snail	[27]
lncRNA MALAT1	-	Wnt/β-catenin	[31]
lncRNA NEAT1	*miR-139-5p*, *β-catenin*	β-catenin/SOX9/TGF-β1	[30]
	miR-129-5p, *PEG3*	NF-κB	[32]
	miR-129-5p, *SOCS2*	-	[33]
	miR-148a-3p, *miR-22-3p*, *Cyth3*	-	[34]
lncRNA Lfar1	-	NF-κB	[35]
lncRNA GAS5	*miR-433-3p*, *TLR10*	NF-κB	[36]
lncRNA-ROR	*miR-6499-3p*	NF-κB	[99]
lncRNA Airn	*EZH2*	KLF2-eNOS-sGC	[98]
lncRNA MEG3	*NLRC5*	-	[37]
lncRNA NORAD	*miR-495-3p*, *S1PR3*	-	[60]
lncRNA XIST	*miR-539-3p*, *ADAMTS5*	-	[44]
lncRNA Mical2	*miR-203a-3p*, *p66Shc*	-	[43]
circRNA608	*miR222*, *PINK1*	Autophagy pathway	[41]
circMTO1	*miR-17-5p*, *Smad7*	-	[24]
circFBXW4	*miR-18b-3p*, *FBXW7*	-	[48]
circCREBBP	*miR-1291*, *LEFTY2*	-	[49]
circ_0071410	*miR-9-5p*	-	[50]

circUbe2k	*miR-149-5p*, *TGF-β2*	-	[59]

ncRNAs: Noncoding RNAs; CAV2: Caveolin-2; TGF-β: Transforming growth factor-β; PI3K: Phosphatidylinositol 3-kinase; AKT: Serine/threonine kinase 1; PPAR: Peroxisome proliferator-activated receptor; PDCD4: Programmed cell death protein 4; AP-1: Activation protein-1; NOX4: Nicotinamide adenine dinucleotide phosphate oxidase 4; Spry1: Sprouty 1; ERK: Extracellular regulated kinase; NF-κB: Nuclear factor κ light chain enhancer of activated B cells; HIF-1α: Hypoxia-inducible factor-1α; VEGF: Vascular endothelial growth factor; TGF-βRI: TGF-β type I receptor; MAPK: Mitogen-activated protein kinase; FGFR: Fibroblast growth factor receptor; JNK: c-Jun N-terminal kinase; ZEB1: Zinc finger E-box binding homeobox 1; PTEN: Phosphatase and tensin homology deleted on chromosome ten; ACSL1: Acyl-CoA synthetase long chain family member 1; HAS2: Hyaluronic acid synthase; EphB2: Erythropoietin-producing hepatocellular receptor B2; SNHG7: Small nucleolar RNA host gene 7; DNMT3A: DNA methyltransferase 3 alpha; PVT1: Plasmacytoma variant translocation 1; ATG: Autophagy-related gene; SCARNA10: Small Cajal body-specific RNA 10; PRC2: Polycomb repressive complex 2; mTOR: Mammalian target of rapamycin; MALAT1: Metastasis-associated lung adenocarcinoma transcript1; NEAT1: Nuclear enriched abundant transcript1; SOX9: SRY-related high mobility group-box gene9; PEG3: Paternally expressed gene 3; SOCS2: Suppressor of cytokine signaling 2; Cyth3: Cytohesin 3; Lfar1: Liver fibrosis associated lncRNA1; GAS5: Growth arrest-special transcript 5; TLR: Toll-like receptor; MEG3: Materally expressed gene 3; NLRC5: NLR Family CARD Domain Containing 5; NORAD: Non-coding RNA activated by DNA damage; S1PR3: Sphingosine 1-phosphate receptor 3; XIST: X-inactive-specific transcript; ADAMTS5: ADAM metallopeptidase with thrombospondin type 1 motif 5; Mical2: Molecule interacting with CasL2; Shc: Src homologous-collagen homologue; PINK1: PTEN-induced putative kinase 1; MTO1: Mitochondrial tRNA translation optimization 1; FBXW4: F-box and WD repeat domain containing 4; CREBBP: CREB binding protein; LEFTY2: Left-right determinant cluster 2; Ube2k:Ubiquitin conjugating enzyme E2 K; STAT3: Signal transducer and activator of transcription 3; Airn: Antisense Igf2r RNA; ROR: Regulator of reprogramming; EZH2: Enhancer of zeste homolog 2; KLF2: Krüppel-like transcription factor 2; eNOS: Endothelial nitric oxide synthase; sGC: Soluble guanylate cyclase.

-induced fibrosis[63]. These findings indicate that elevated miR-29 Level in serum may be a promising biomarker for diagnosis of liver fibrosis[63]. Another set of biomarkers (NIS4) consisting of miR-34a-5p, α-2 macroglobulin, YKL-40 and glycated hemoglobin have been developed to successfully identify patients who have a higher risk of disease progression with non-alcoholic fatty liver disease and liver fibrosis. The diagnostic value of the NIS4 algorithm was not affected by age, gender, BMI and transaminase[64]. Similarly, Azar *et al*[65] constructed a miRNA regulatory network using bioinformatics tools and identified five upregulated miRNAs (miR-21-5p, miR-222-3p, miR-221-3p, miR-181b-5p, and miR-17-5p) that targeted tissue inhibitor of metalloproteinase 3 in activated HSCs, and these results have been verified in a mouse model of liver fibrosis. Zhang *et al*[66] performed a logistic regression analysis to show that miR-1225-3p, miR-1238, miR-3162-3p, miR-4721, and miR-H7 could distinguish, with high sensitivity and specificity, nonsignificant fibrosis from significant fibrosis in chronic hepatitis B (CHB) patients. Some researchers screened miRNAs in serum from HCV-related liver fibrosis patients and found that miR-484 was significantly downregulated in advanced liver fibrosis compared to early liver fibrosis and liver cancer[67], which indicates that miR-484 may be used as a biomarker for staging liver fibrosis in patients with HCV. Besheer *et al*[68] performed diffusion-weighted magnetic resonance imaging of livers in patients with liver fibrosis caused by chronic hepatitis C and compared the apparent diffusion coefficient (ADC) with miRNA expression pattern in liver biopsies. They found that ADC was closely associated with expression of miR-200b, miR-21, and miR-29, and the accuracy of ADC combined with miR-200b to distinguish early and late liver fibrosis was 80.2%[68]. In a discovery cohort of 183 patients with non-alcoholic fatty liver disease, scientists identified that plasma miR-193a-5p was consistently maintained at a high level and was closely associated with grade of fibrosis, which was verified in a cohort of 372 additional cases[69]. Results from another study confirmed that miR-103a-3p and miR-425-5p were stably expressed in exosomes of serum derived from mice and humans infected with schistosomiasis[70]. MiR-146a-5p could distinguish mild (grades 0 and I) and severe fibrosis (grades II and III) and could be used for staging liver fibrosis[70].

LncRNAs are useful in the diagnosis of liver fibrosis. A study compared lncRNAs profiles of serum exosomes from patients with liver fibrosis and healthy controls and found that the expression level of lncRNA MALAT1 was significantly increased in the serum of fibrotic patients[31]. Serum lncRNA GAS5 was significantly upregulated in patients with advanced liver fibrosis compared with nonfibrotic patients[71]. Serum lncRNA-p21 had 70% specificity and 100% sensitivity in diagnosing liver fibrosis in patients with CHB[72]. LncRNA SCARNA10 was higher in liver and serum samples in patients with advanced liver fibrosis compared with healthy controls[23].

In addition to miRNAs and lncRNAs, circRNAs have shown differential expression in patients with liver fibrosis. The expression level of circRNA death inducer-obliterator 1 (circDIDO1) was decreased in serous exosomes derived from patients with liver fibrosis[73], while serum circMTO1 was negatively correlated with the degree of liver fibrosis in patients with CHB[24]. All these findings suggest that ncRNAs have potential as novel noninvasive biomarkers for the diagnosis and staging of liver fibrosis with high sensitivity and specificity.

POTENTIAL APPLICATION OF NCRNAS FOR THE TREATMENT OF LIVER FIBROSIS

Early liver fibrosis is deemed to be reversible. When the injury is removed, activated HSCs (myofibroblasts) are reduced through deactivation or apoptosis to slow down the fibrotic process and even lead to

regression. Studies have shown that patients with chronic hepatitis B or chronic hepatitis C have reduced liver fibrosis after receiving antiviral therapy[74]. In addition, therapies such as antioxidants, renin–angiotensin system inhibitors, and traditional Chinese medicine[75] are also considered promising for treatment of liver fibrosis, although more clinical trials are needed to confirm their safety and efficacy. As extensive cytokines and signaling pathways are involved in the pathogenesis and progression of liver fibrosis, ncRNA-based therapies that target various signaling pathways are being developed based on the outstanding gene silencing effect of miRNAs and the sponging effect of lncRNAs and circRNAs.

With strong inhibitory effects on a variety of fibrotic diseases such as myocardial fibrosis[76], pulmonary fibrosis[77], and renal fibrosis[78], miR-29 families are regarded as a potential therapeutic target for fibrosis. Yang *et al*[79] reported that miR-29a reduced liver fibrosis and ECM by directly targeting PI3KP85α in cholestatic liver fibrosis, and this supports the potential of miR-29a for the treatment of liver fibrosis. However, recent studies showed that, even though upregulation of miR-29 inhibited fibrosis, it could also lead to type 2 diabetes and insulin resistance[80]. Researchers have assessed the therapeutic effect of a synthetic miR-223 analog in a murine NASH model and have found that miR-223 treatment inhibited HSC activation through the downregulation of transcription of proinflammatory cytokines and chemokines together with NOD-like receptor 3 (NLRP3) inflammasome [81]. In addition, miR-223 was reported to inhibit the activation and proliferation of HSCs by targeting Gliotactin family zinc finger 2 (GLI2) and PDGFRα/β in CCl_4-induced liver fibrotic mice[82]. These studies clearly demonstrate the potential of miR-223 as a therapeutic strategy for liver fibrosis, although the underlying mechanisms vary.

Exosomes are small vesicles that are stable in body fluids, low in immunogenicity, can be engulfed by cells, and have been used as delivery vectors for easily degradable molecules such as RNA to treat diseases like liver cancer[83]. Exosomes have also been explored in treating liver fibrosis. Gao *et al*[84] found that miR-690 produced by Kupffer cells could be delivered to HSCs by exosomes to inhibit fibrosis by targeting nicotinamide adenine dinucleotide kinase. In a murine NASH model, miR-690 mimics decreased liver fibrosis markers and alleviated NASH phenotypes significantly. Exosomal miR-223 derived from natural killer cells has also been shown to target ATG7 in HSCs by inhibiting autophagy, leading to reduced fibrosis[85]. In addition, mesenchymal stem cell (MSC)-derived exosomes have been well studied as a promising treatment option for liver fibrosis[86]. Human bone MSCs (hB-MSCs)-derived exosomal miR-618[87] and human tonsil-derived MSCs (hT-MSCs)-derived exosomal miR-486[88] have been shown to alleviate liver fibrosis by targeting *Smad4* and smoothened (*Smo*) genes, respectively. MiR-6766-3p derived from 3D-cultured human embryonic stem cells were enriched in exosomes and attenuated TGFβ1/SMADs by targeting TGFβRII to inhibit proliferation of HSCs[89]. In another study, adipose-derived stromal cells were transfected with miR-150, and the culture supernatants were collected to treat HSCs or infuse into mice with liver fibrosis. Expression of several fibrosis markers such as Collagen 1A1 and α-smooth muscle actin (α-SMA), as well as the levels of systemic inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α were significantly decreased in miR-150-treated mice compared with the control group[90]. This indicates that the exosomal miR-150 has antifibrotic activity through targeting of the TGF-β pathway. Zhou *et al*[91] co-cultured HSCs with human umbilical cord MSCs and found that expression of miR-148a-5p in HSCs was significantly upregulated, which decreased liver fibrosis by inhibiting Notch2 *in vivo* and *in vitro*.

Bone marrow MSCs have also been shown to reduce liver fibrosis by altering expression of lncRNAs. One such example comes from lncRNA BIHAA1 derived from bone marrow MSC-treated HSCs. Bone marrow MSCs inhibited liver fibrosis by lncRNA BIHAA1 targeting miR-667-5p[92]. Sun *et al*[93] found that silencing lncRNA SNHG promoted differentiation of bone marrow MSCs into hepatocyte-like cells and reduced cirrhosis through the miR-15a/Smad ubiquitin regulatory factor 1 (SMURF1)/UV radiation resistance associated gene (UVRAG)/ATG5/Wnt5a axis.

CircRNAs have also been investigated to treat liver fibrosis. Ma *et al*[73] reported that circDIDO1 in exosomes derived from MSCs regulated the PTEN/AKT pathway by sponging miR-141-3p, thereby inhibiting activation of HSCs and reducing expression of α-SMA and Collagen I to alleviate liver fibrosis. Similarly, MSC-derived exosomal circRNA cyclin dependent kinase 13 (circCDK13) inhibited activation of PI3K/AKT and NF-κB signaling pathways to reduce liver fibrosis by regulating miR-17-5p and its target gene K (lysine) acetyltransferase 2B (KAT2B)[94].

Delivery systems are one of the key issues to be resolved in order to protect ncRNAs from being degraded. Lipid nanoparticles (NPs) for ncRNAs delivery have been developed. Hu *et al*[95] encapsulated miR-30a-5p and an antifibrotic peptide Relaxin into NPs and injected them into fibrotic mice. NPs increased the exosomal miR-30a-5p level, which in turn reversed the activated HSCs into a quiescent state by targeting liver macrophages[95]. Furthermore, NPs encapsulated with miR-29b and Germacrone, a major component of the traditional Chinese medicine *Rhizoma curcuma*, have been shown to have robust antifibrotic activity *in vitro* and *in vivo*[96]. The ncRNAs showed the potential for the treatment of liver fibrosis are collected in Table 2.

Table 2 Noncoding RNAs for the potential treatment of liver fibrosis

ncRNAs	Target genes	Signaling pathways	Ref.
miR-29a	*PI3KP85α*	PI3K/AKT	[79]
	Fstl	TGF-β/Smad2, JNK	[21]
miR-223	*NLRP3* inflammasome	NOD signaling pathway	[81]
	GLI2, PDGFRα/β	Hedgehog, PDGF	[82]
hB-MSCs-derived exo-miR-618	*Smad4*	TGF-β/Smad2	[87]
3DhESCs-derived exo-miR-6766-3p	*TGFβRII*	TGF-β/Smad	[89]
hT-MSCs-derived exo-miR-486	*Smo*	Hedgehog/GlI2	[88]
NK cells-derived exo-miR-223	*ATG7*	Autophagy pathway	[85]
KCs-derived exo-miR-690	*NADK*	-	[84]
lncRNA SNHG	*miR-15a, MURF1*	UVRAG/ATG5/Wnt5a	[93]
lncRNA BIHAA1	*miR-667-5p*	-	[92]
MSCs-derived exo-circDIDO1	*miR-141-3p*	PTEN/AKT	[73]
MSCs-derived exo-circCDK13	*miR-17-5p, KAT2B*	PI3K/AKT, NF-κB	[94]

ncRNAs: Noncoding RNAs; PI3K: Phosphatidylinositol 3-kinase; AKT: Serine/threonine kinase 1; Fstl: Follistatin-like 1; TGF-β: Transforming growth factor-β; JNK: c-Jun N-terminal kinase; NLRP3: NOD-like receptor family, pyrin domain containing 3; Smo: Smoothened; GLI2: Gliotactin family zinc finger 2; PDGFR: Platelet-derived growth factor receptor; MSC: Mesenchymal stem cell; hB-MSC: Human bone MSC; hT-MSC: Human tonsil-derived MSC; 3DhESC: 3D-cultured human embryonic stem cells; TGF-βRII: TGF-β type II receptor; ATG: Autophagy-related gene; KCs: Kupffer cells; NADK: NAD kinase; SNHG: Small nucleolar RNA host gene; SMURF1: Smad ubiquitin regulatory factor 1; UVRAG: UV radiation resistance associated gene; DIDO1: Death inducer-obliterator 1; PTEN: Phosphatase and tensin homology deleted on chromosome ten; CDK13: Cyclin dependent kinase 13; KAT2B: K (lysine) acetyltransferase 2B.

CONCLUSION

It is well known that persistent liver fibrosis leads to irreversible fibrosis, decompensated cirrhosis, and even HCC, which emphasizes the importance of treatment during early-stage fibrosis to prevent disease progression. Therefore, it is important to diagnose liver fibrosis before clinical symptoms appear. Although liver biopsy is considered the gold standard for the diagnosis of liver fibrosis, its invasive nature limits its clinical use, especially in early disease stages. Although some ncRNAs are closely associated with the pathogenesis and progression of liver fibrosis, there is still insufficient evidence for diagnosing and staging liver fibrosis using ncRNAs alone. Some studies have suggested combining ncRNAs with other indicators (biomarkers) in blood or with imaging techniques to increase the accuracy of liver fibrosis diagnosis. There is no specific anti-hepatic fibrosis drug in clinical use, although several candidates have already been enrolled in clinical trials. The main strategy for antifibrosis therapy is to treat the etiology and alleviate liver inflammation. NcRNAs are able to target various inflammation-related signaling pathways to reduce liver fibrosis. The latest studies have found that miR-20b-5p[97] and lncRNA Antisense Igf2r RNA (lncRNA Airn)[98] can inhibit HSCs activation to alleviate liver fibrosis process. Salvianolic acid B treatment relieved the activation of HSCs through decreasing the expression of lncRNA regulator of reprogramming (lncRNA-ROR)[99], which providing new targets for the treatment of liver fibrosis. Although most of these findings are based on *in vitro* studies, and therefore, need validation *in vivo*. With the rapid progress of techniques such as gene editing, NP-based delivery systems, and synthetic biology, MSC-derived exosomal ncRNAs may become promising treatment options for liver fibrosis in the near future.

FOOTNOTES

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MINIREVIEWS

Approach to thromboelastography-based transfusion in cirrhosis: An alternative perspective on coagulation disorders

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Abstract

Viscoelastic tests, specifically thromboelastography and rotational thromboelastometry, are increasingly being used in the management of postoperative bleeding in surgical intensive care units (ICUs). However, life-threatening bleeds may complicate the clinical course of many patients admitted to medical ICUs, especially those with underlying liver dysfunction. Patients with cirrhosis have multiple coagulation abnormalities that can lead to bleeding or thrombotic complications. Compared to conventional coagulation tests, a comprehensive depiction of the coagulation process and point-of-care availability are advantages favoring these devices, which may aid physicians in making a rapid diagnosis and instituting early interventions. These tests may help predict bleeding and rationalize the use of blood products in these patients.

Key Words: Bleeding; Chronic liver disease; Cirrhosis; Thromboelastography; Viscoelastic tests

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Core Tip: Viscoelastic hemostatic assays are increasingly used as "point-of-care" tests, providing real-time, dynamic insight into the complex coagulation aberrations seen in cirrhotic patients. In cirrhosis, all patients undergoing a high-risk invasive procedure or who are actively bleeding should undergo thromboelastography (TEG) on initial evaluation, if this testing is available. Any reasonable TEG-based strategy will likely represent an improvement over strategies using traditional coagulation tests. The best approach will be to use TEG supplemented by standard platelet count and fibrinogen testing. TEG is a promising diagnostic modality and may help in predicting bleeding and aid in the rationalization of the use of blood products in these patients.

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INTRODUCTION

The liver is essential in maintaining hemostasis[1]. Patients with cirrhosis may demonstrate altered coagulation and are often considered "auto-anticoagulated"[2]. However, the current understanding of coagulopathy is that patients with cirrhosis have a rebalanced coagulation status[3]. This balance is precarious due to alterations in the hepatic synthesis of pro- and anticoagulant factors. The resilience of the hemostatic system can be further decreased in cirrhotic patients by acute clinical conditions like systemic infection, altered volume status, and impaired renal function.

Given our current understanding of coagulation status in cirrhosis patients, there is considerable interest in tests of coagulation that could provide a truly global view of the coagulation system. Conventional coagulation tests (CCTs), like prothrombin time (PT) and activated partial thromboplastin time (aPTT), are indicators of general liver dysfunction. However, these tests fail to depict the totality of *in vivo* coagulation dysfunction, and lack insight into factors such as blood flow dynamics, endothelial tissue factor (TF), platelet function. They are also limited in their ability to aid in the decision of whether to administer plasma or whole blood[4,5]. Despite such concerns, these CCTs are commonly used to drive clinical decisions.

Thromboelastography (TEG) provides a more physiologically accurate assessment of the coagulation system. TEG has been used effectively as a rapid point-of-care test to assess hypercoagulable, hypocoagulable, and rebalanced coagulation states to evaluate blood transfusion requirements, suggest whether anticoagulation is required, and, if so, aid in the selection of anticoagulant therapy[6].

However, the ideal strategy for using TEG to guide the determination of blood product transfusion is unclear. Although the literature is replete with prospective data demonstrating the superiority of TEG over CCTs for non-surgical patients in terms of the requirement of blood transfusion, a mortality benefit has not been established[7-9]. The present article aims to review the current evidence supporting the use of TEG and the clinical significance of this testing modality in the guidance of blood transfusion in cirrhosis patients.

HEMOSTATIC SYSTEM IN LIVER DISEASE

Per the cell-based model of hemostasis, coagulation occurs not as a "cascade" but in 3 overlapping stages: (1) The initiation phase ensues on tissue factor (TF)-carrying cells. If the procoagulant stimulus is sufficiently strong, factors Xa, IXa, and thrombin are formed in adequate levels to initiate the coagulation process; (2) The amplification phase occurs as the activity moves from the TF-carrying cell to the platelet surface. The procoagulant stimulus is intensified causing platelets to attach, activate, and hoard activated cofactors on their surfaces; and (3) The propagation phase in which the "tenase" and "prothrombinase" complexes gather on the platelet surface and generate the large amounts of thrombin necessary to form a hemostatic fibrin clot[10].

In cirrhosis, all three phases are limited by hepatic synthetic dysfunction and portal hypertension, resulting in a delicate state of "new equilibrium" (Figure 1)[11]. However, this balance can be altered by concomitant conditions such as sepsis or acute kidney injury (AKI) as a result of the interaction between platelets and released inflammatory mediators (Figure 2). Thus, the coagulation profile in cirrhotic patients is dynamic, with possible resolution of global coagulation deficiencies once the acute critical illness resolves. The cell-based model of coagulation also explains why regional hemostatic changes at an injury site do not override the systemic hemostatic equilibrium. Accordingly, CCTs may remain unchanged in patients with liver dysfunction, even with clinically evident bleeding.

According to Hoffman's concept of the cell-based coagulation model, bleeding can arise from disorders of primary hemostasis (abnormal platelet plug formation) or secondary hemostasis (reduced thrombin generation and subsequent fibrin clot formation). The liver plays a critical role in maintaining both primary and secondary hemostasis[11]. In fact, the liver is the site of synthesis of most coagulation factors, with the exception of von Willebrand factor (vWF), factor VIII (only partly synthesized in the liver), and calcium[12].

Bleeding complications in cirrhotic patients may occur due to hemostatic failure or non-hemostatic causes. The term "spontaneous hemostasis-related bleeding" has recently been introduced to distinguish bleeding due to hemostatic anomalies from that related to portal hypertension, trauma, or peptic ulcers. It is defined as an unprovoked hemorrhage of unexplained cause. However, it should be emphasized that spontaneous bleeding is uncommon in patients with cirrhosis, and bleeding is typically

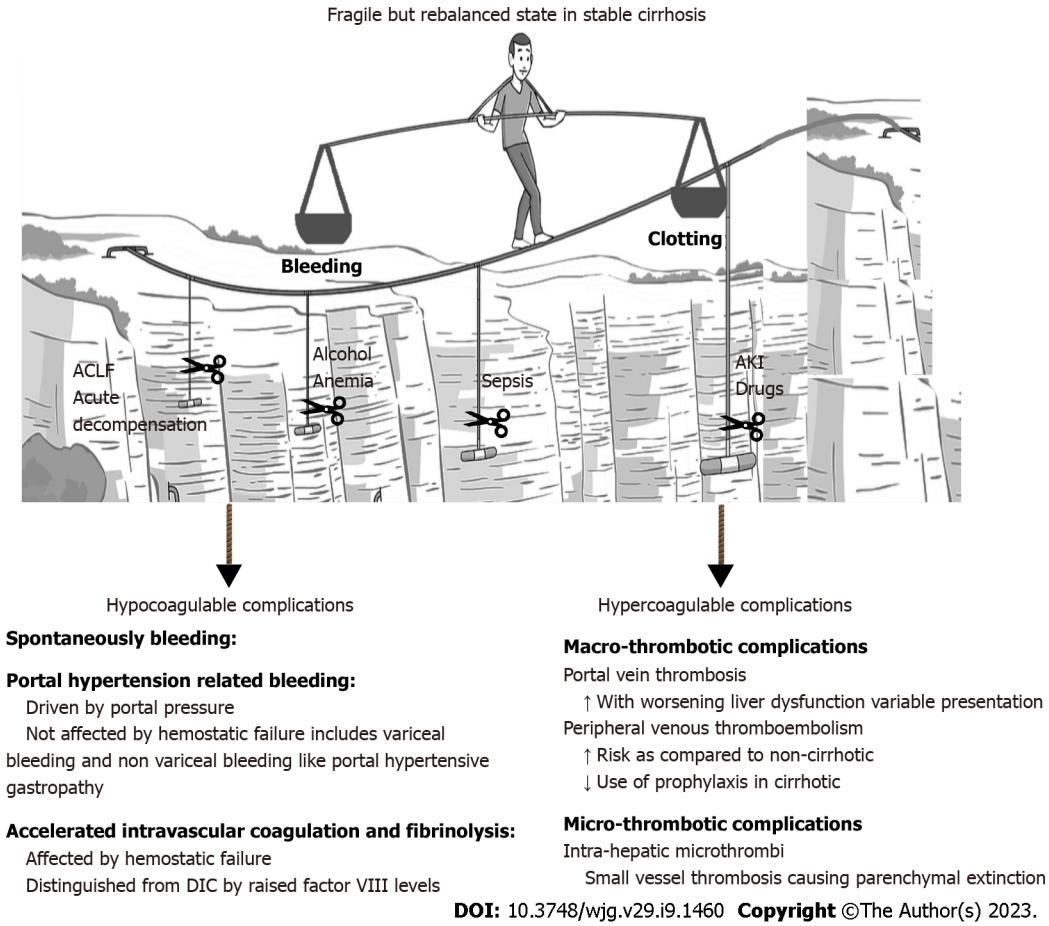


Figure 1 Rebalanced hemostasis in cirrhosis. ACLF: Acute-on-chronic liver failure; AKI: Acute kidney injury; DIC: Disseminated intravascular coagulation.

related to portal hypertension caused by increased portal pressure rather than hemostatic failure. This was conclusively demonstrated by the inability of recombinant factor VII to achieve better control of variceal rebleeding[13,14]. Notably, a bleed not primarily caused by hemostatic failure can evolve into a hemostatic bleed due to severe blood loss and consumptive coagulopathy. Bleeding (tertiary hemostasis disorder) can also be due to premature platelet or fibrin clot dissolution or excessive fibrinolysis, which in cirrhotic patients has been termed "accelerated intravascular coagulation and fibrinolysis" (AICF). AICF manifests as mucosal or puncture wound bleeding, and the pathophysiology of this disorder is not entirely understood. Hyperfibrinolysis parallels the severity of liver disease: mild systemic fibrinolysis is encountered in 30%-45% of cirrhotic patients, with clinically detectable fibrinolysis in only 5%-10%. AICF can be distinguished from disseminated intravascular coagulation by increased factor VIII levels (Figure 1)[15,16]. The 3 phases of coagulation in liver disease resulting in a "rebalancing" of hemostasis are summarized in Table 1[17,18].

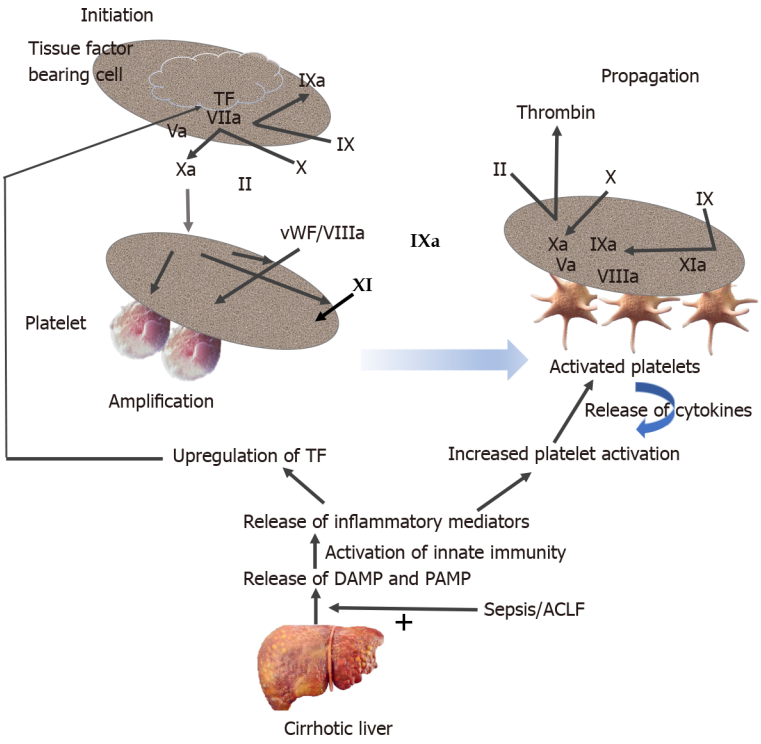
For the past several decades, bleeding has been a major concern in the management of cirrhotic patients. However, thrombotic complications are being increasingly acknowledged and are attributed to shifts in hemostatic balance. In one case-control study, the relative risk of venous thromboembolism (VTE) in patients with cirrhosis was 1.74 (95%CI: 1.54-1.95)[19]. These conclusions were mirrored in a study by Wu *et al*[20], which showed an increased likelihood of VTE in cirrhosis [odds ratio (OR) 1.23 in compensated cirrhotic patients; OR 1.39 in decompensated cirrhotic patients]. Dysfibrinogenemia (*i.e.* altered fibrinogen) may result in decreased permeability of the formed clot, as well as other factors that contribute to coagulopathy. It may even confer hypercoagulable features, manifesting as macro- and micro-thrombotic complications. A hypercoagulable state also frequently occurs in cirrhosis patients due to concomitant primary biliary cholangitis, non-alcoholic fatty liver disease, or primary sclerosing cholangitis[21].

The most common macro-thrombotic presentation in liver disease is portal vein thrombosis (PVT), occurring in 8% to 18% of cirrhosis patients[18]. The incidence of PVT increases with deteriorating liver function and decreased portal flow. Deep venous thrombosis and pulmonary embolism (PE) are other macro-thrombotic complications, which have been reported in 5% of hospitalized patients with chronic liver disease (CLD)[17,22]. Micro-thrombotic complications include intrahepatic microthrombi ("parenchymal extinction"), resulting in nodules, porto-pulmonary hypertension, and cirrhosis arising as an ischemic/reinjury process. These complications often merit exigent consideration of anticoagulant

Table 1 Three phases of coagulation in liver disease

Hemostasis stage	Hypocoagulable state	Hypercoagulable state
Primary hemostasis: Platelet activation and interaction with injured endothelium	Thrombocytopenia: (1) Decreased amount: Splenic sequestration, decreased thrombopoietin levels, bone marrow suppression, autoantibody destruction; and (2) Poor function: Uremia, changes to the vessel wall phospholipid composition, anemia (Hgb < 7 g/dL), decreased margination	Low levels of ADAMTS-13; Increased levels of vWF; Increased number of activated platelets
Secondary hemostasis: Fibrin clot formation	Low levels of factors II, V, VII, IX, X, and XI; Low levels of fibrinogen; Vitamin K deficiency (malabsorption in cholestatic disorders)	Elevated levels of factor VIII; Decreased levels of proteins C and S; Decreased levels of antithrombin, and heparin cofactor II
Fibrinolysis	Accelerated intravascular coagulation and fibrinolysis: (1) Low levels of factor XIII and thrombin-activated fibrinolysis inhibitor; (2) Elevated levels of tPA; (3) Decreased level of α2-antiplasmin; and (4) Dysfibrinogenemia	Low plasminogen levels; Dysfibrinogenemia; High plasminogen activator inhibitor

Hgb: Hemoglobin; tPA: Tissue plasminogen activator; vWF: von Willebrand factor.



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Figure 2 Dynamic coagulation profile in cirrhosis. ACLF: Acute-on-chronic liver failure; DAMP: Damage-associated molecular patterns; PAMP: Pathogen-associated molecular patterns; TF: Tissue factor.

usage.

TESTS OF COAGULATION IN CIRRHOSIS

As of this article, all available laboratory hemostasis measures have significant limitations when applied to patients with liver disease. The paradigm of this phenomenon is the cirrhotic patient, for which PT and international normalized ratio (INR) were developed to monitor warfarin-treated patients by measuring the activity of an added commercially available thromboplastin reagent. PT and aPTT indicate the onset of thrombin generation; however, they do not reflect enzymatic coagulation. PT/INR has been validated as a prognostic marker for mortality in liver disease, but has never been validated to predict bleeding risk or guide transfusion of blood products, especially for pre-procedure risk mitigation[14]. Nonetheless, this measure has been used for decades as a surrogate for bleeding risk in cirrhosis despite the fact that the arbitrary cut-off points used as clinical targets for the prevention of bleeding are not supported by scientific evidence. Furthermore, using fresh frozen plasma (FFP) to

normalize a raised INR in cirrhosis does not alter thrombin (factor II) production, but exacerbates portal hypertension[23-25].

Thrombocytopenia is the most common hematological abnormality in patients with liver disease. Platelet count thresholds are often specified for invasive procedures in patients with severe cirrhosis-related thrombocytopenia. In vitro data suggest that a threshold of 50-55 × 10^9/L is necessary for adequate platelet activity, and levels below this range fail to promote thrombin generation[26]. However, the platelet function associated with primary hemostasis (*i.e.* adhesiveness and aggregation) has not been evaluated. Current guidelines and expert opinion recommend considering platelet-raising treatments before high-risk procedures, or in patients with active bleeding with platelet counts < 50 × 10^9/L. However, there is no firm evidence that prophylactic platelet transfusion to achieve this target enhances hemostasis[15,23].

As mentioned previously, platelet count alone does not account for other factors affecting platelet function in cirrhosis[27]. For example, uremic platelet dysfunction (*e.g.*, hepatorenal syndrome) can result in impaired platelet activity with decreased serotonin in alpha granules and dysregulated metabolism of thromboxane A2. Anemia can also affect platelet function. In patients with hematocrit < 25%, erythrocyte concentration is inadequate to facilitate platelet margination, impairing the clotting process. Sepsis and endotoxemia due to bacterial translocation also can affect platelet function.

Recently, fibrinogen levels have replaced INR to couple with platelet count in the evaluation of bleeding risk. The Clauss method for detecting fibrinogen is turbidimetric and relies on thrombin-induced fibrin formation. Nevertheless, fibrinogen levels do not account for the synthesis of abnormal fibrinogen in cirrhotic patients caused by hypersialylation of the fibrinogen, leading to impaired fibrinogen-to-fibrin conversion[28]. In trauma surgery patients without underlying liver disorder, administration of fibrinogen factor to accomplish levels of fibrinogen > 200 mg/dL is associated with improved hemostasis. However, in routine clinical practice, the most agreed-upon cut-off for fibrinogen in cirrhotic patients with active bleeding is > 120 mg/dL[29]. In cirrhotic patients, spontaneous or procedure-related bleeding is relatively common when plasma fibrinogen levels are less than 100 mg/dL. Whether this relationship is causal or reflects disease severity is unclear. As such, the available evidence suggests that tests measuring clot formation and strength (*i.e.* fibrinogen) may have better predictive value for bleeding events than coagulation initiation tests[29,30].

Primary hyperfibrinolysis is an increasingly vital pathophysiological process in CLD, resulting in an increased risk of variceal bleeding. D-dimer is a nonspecific marker of fibrin degradation. While evidence suggests that elevated D-dimer indicates hyperfibrinolysis and can predict gastrointestinal bleeding in this population, elevated D-dimer alone provides limited information regarding an individual's fibrinolytic state[31,32].

Thrombin generation assays (TGAs) evaluate the time of thrombin generation and its decline when plasma is triggered by TF and phospholipids. Thus, TGA can reflect the activity of both pro- and anticoagulant factors[33,34]. Nevertheless, clinical trials are needed to test this conjecture. Similar to PT and aPTT, TGA is performed on plasma rather than whole blood. However, because of their method, TGAs approximate the *in vivo* coagulation balance better than CCTs.

TEG quantitatively assesses the capability of whole blood to form a clot, providing a comprehensive picture of coagulation status compared to standard laboratory tests, which are confined to developing the first fibrin strands. However, TEG is insensitive to the platelet adhesion and aggregation activity of vWF and the anticoagulant actions of protein C and protein S; therefore, it may lead to an underestimation of hemostatic capacity[17] (Table 2).

PRINCIPLES OF TEG

The principle of the *in vitro* TEG test is to detect and quantify dynamic changes in the viscoelastic properties of whole blood during clotting under low shear stress (Figure 3A). TEG results are depicted as 2-dimensional graphs, with time on the x-axis and amplitude (in millimeters) on the y-axis (Figure 3B). A normal TEG trace appears similar to a cognac glass lying on its side (Figure 4)[17]. An evident prolongation of R is associated with clotting factor levels of 30% or less[35]. Different activators can be added to the blood to better assess various aspects of the clotting cascade (Table 3). Conventional TEG involves clot initiation by adding kaolin, simulating the intrinsic coagulation pathway. In contrast, rapid TEG involves the addition of kaolin and TF, causing massive thrombin burst and providing initial results (K time) within 6 min and alpha angle/MA within 15 min[36,37]. Thus, the results of rapid TEG can be achieved approximately 10 min earlier than the kaolin TEG and about 30 min earlier than CCTs [37]. This could guide critical resuscitations more competently, enabling real-time monitoring and goal-directed therapy. Though the activators reduce the test turnaround time (*e.g.*, kaolin), the sensitivity of viscoelastic tests (VETs) could be blunted, and subtle changes in coagulation and clot lysis might not be detected[17] (Table 4).

Correlation of CCTs and VETs

A strong correlation between TEG measures of clot formation and clot strength and conventional

Table 2 Thromboelastography components and their clinical implications

Nomenclature	Definition	Function	Significance	Most closely related CCT
Reaction time or R-time	Time (min) to reach an amplitude of 2 mm	Clot initiation	Informs about enzymatic reaction leading to thrombin and fibrin generation. Increased R-time, factor deficiency or reduced function, resulting in hypocoagulability; Shortened R-time, factor hypercoagulability	PT and aPTT
K-time	Time (min) from 2-20 mm amplitude	Clot kinetics	Depicts rate of clot development–fibrin polymerization, cross-linking, and platelet interaction. Long K-time, hypocoagulability; Short K-time, hypercoagulability	Fibrinogen level and platelet count
Angle or α	Slope between R and K	Clot kinetics	Also depicts the kinetics of clot development. Low-angle, hypocoagulability; High-angle, hypercoagulability	
MA	Highest level of amplitude achieved by the clot	Clot strength	Provides assessment of overall clot strength	Platelet count and fibrinogen levels
Coagulation index	Composite indicator of coagulation profile		A linear combination of the above parameters serving as a global view of the patient's hemostatic profile. Increased in hypercoagulable states; Decreased in hypocoagulable states	
LY30	Degree of lysis (%) 30 min after MA is reached	Clot stability	Measure of fibrinolysis. Above normal LY30 suggests hyperfibrinolysis	No equivalent test

aPTT: Activated partial thromboplastin time; CCT: Conventional coagulation test; MA: Maximum amplitude; PT: Prothrombin time.

Table 3 Procedural bleeding risk in patients with cirrhosis

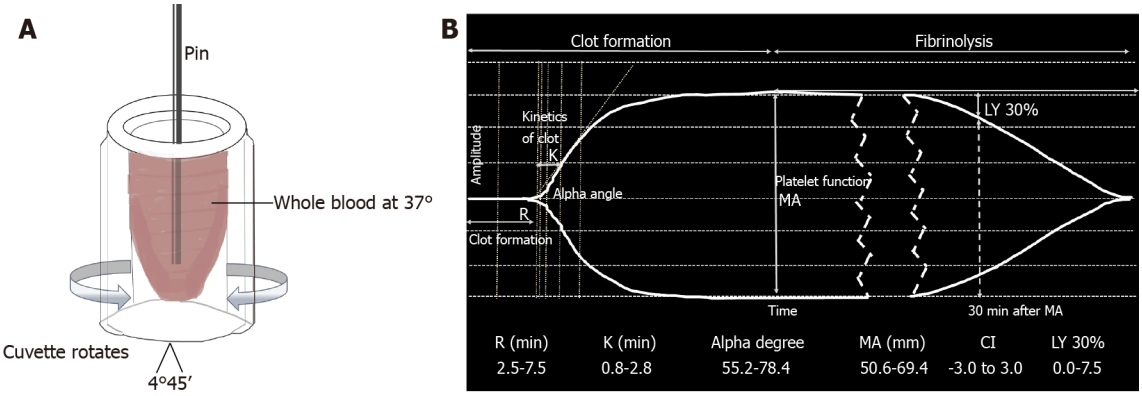
High-risk procedures	Intermediate-risk procedures	Lower-risk procedures
Intrabdominal/orthopedic/cardiac surgery	Percutaneous endoscopic gastrostomy	Paracentesis
Brain or spinal surgery	Percutaneous or transjugular liver biopsy	Thoracentesis
Intracranial catheter insertion	Transjugular intrahepatic portosystemic shunt	Central line placement
Endoscopic mucosal resection or endoscopic submucosal dissection	Endoscopy (*e.g.*, percutaneous gastrostomy placement, cystogastrostomy, biliary sphincterotomy)	Endoscopy (*e.g.*, diagnostic, variceal ligation, uncomplicated polypectomy)
Complicated polypectomy	Percutaneous biopsy of extra-hepatic organ or lesions	Cardiac catheterization
Natural orifice transluminal endoscopic surgery	Trans-arterial or percutaneous hepatocellular carcinoma therapies	Hepatic venous pressure gradient measurement
	Lumbar puncture	

Table 4 Various types of thromboelastography assays

TEG channel	Activator	Function
Native TEG	None	Theoretically most sensitive to subtle coagulopathic changes and hyperfibrinolysis
Conventional TEG	Kaolin	Activates clotting cascade to expedite results
Rapid TEG	Tissue factor + kaolin	Activates clotting cascade to expedite results
Functional fibrinogen TEG	Glycoprotein IIb/IIIa inhibitor	Inhibits platelets to isolate the contribution of fibrinogen
Heparinase TEG	Heparinase	Inhibits heparin; the presence of heparin (endogenous or exogenous) is suggested when this channel shows improved clotting compared to other channels

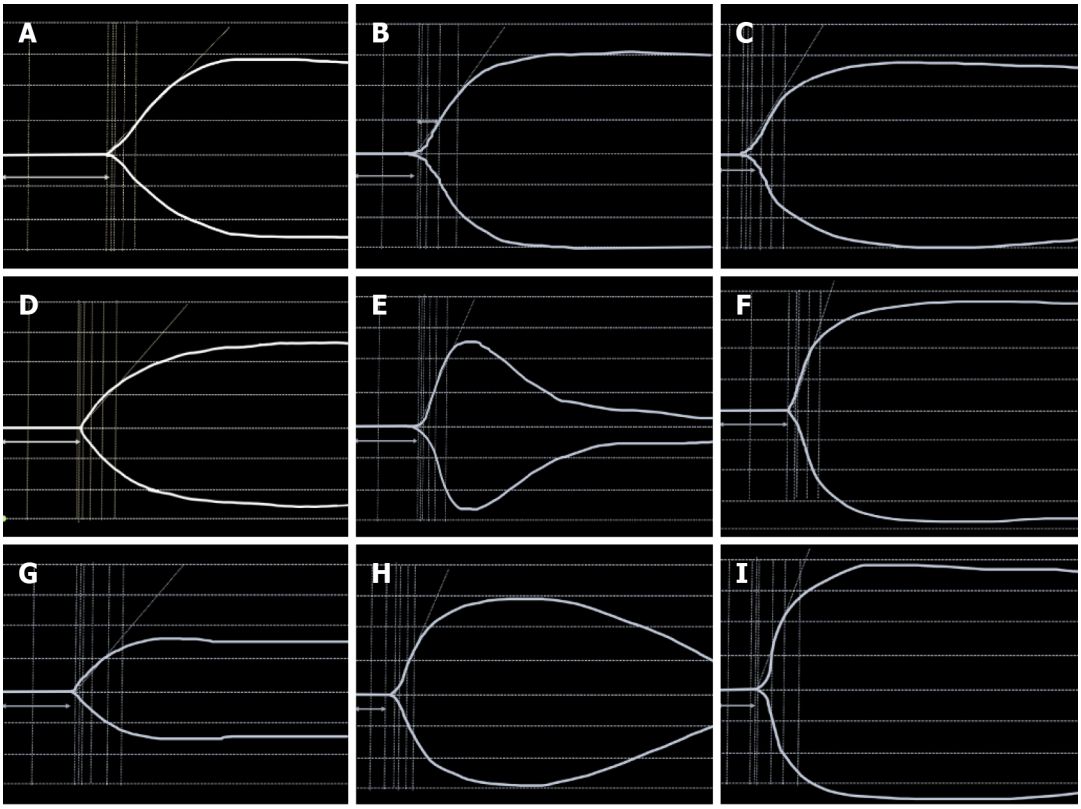
TEG: Thromboelastography.

fibrinogen level has been observed in CLD patients who are critically ill. Nevertheless, weak or unpredictable correlations exist between TEG and CCTs in measuring coagulation initiation (*i.e.* TEG R-time and PT/INR/aPTT), TEG and conventional platelet count, and measures of fibrinolysis (TEG LY30 and traditional D-dimer)[38-40]. The absence of a correlation between PT/INR and R may be explained by several elements, such as the use of different activators, the use of whole blood *vs* plasma, and the



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Figure 3 Basis and results of the thromboelastography. A: Basis of the thromboelastography (TEG) test; B: TEG tracing and relevant parameters (kaolin-activated). MA: Maximum amplitude.



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Figure 4 Tracing of thromboelastography in various clinical conditions. A: Low clotting factors; B: Normal trace; C: Enzymatic hypercoagulability; D: Low fibrinogen levels; E: Primary fibrinolysis; F: Platelet hypercoagulability; G: Low platelet function; H: Secondary fibrinolysis; I: Enzymatic and platelet hypercoagulability.

fact that R-time, unlike INR, reflects the balance of both pro- and anticoagulant factors. This supports the evidence that clotting initiation and speed measures are challenging to interpret in this cohort, while TEG maximum amplitude (MA) and conventional fibrinogen may be more reliable. Nonetheless, the results of these tests should always be correlated with the clinical situation.

CLINICAL APPLICATIONS OF TEG IN LIVER DISEASE

TEG and invasive procedures in patients with cirrhosis

Bleeding complications after invasive procedures are always a concern in cirrhotic patients, though the incidence varies widely[41]. Although the risk of bleeding after the procedure is related to alterations in

clotting factors, the risk is also inherent to a given procedure (Table 3) and the given clinical situation [41]. In cirrhotic patients with acute illness or acute-on-chronic liver failure, the association between clotting tests and bleeding may not be as apparent or evident as in stable patients. Moreover, managing complications, such as sepsis or AKI, instead of correcting hemostatic abnormality, may result in improved outcomes. A retrospective study revealed that AKI was the only independent risk factor for post-paracentesis hemoperitoneum. In contrast, no significant difference was observed in CCTs (platelet count and INR levels) between patients with or without this complication[42].

Three recent randomized trials conducted in cirrhotic patients undergoing invasive procedures demonstrated a decreased requirement for prophylactic blood product transfusions using TEG-guided transfusions compared to standard test-based protocols[7-9]. However, they could not demonstrate any relationship between abnormal TEG tracing and bleeding, primarily due to the scarcity of documented bleeding events. Similarly, TEG did not help to predict the inability to control bleeding or prevent rebleeding. Also, no impact on other clinically relevant outcomes was observed. Moreover, each study used a different transfusion protocol, making it difficult to know whether the lower cutoff for transfusion would have been more beneficial. In another study of cirrhotic patients undergoing various invasive procedures without prophylactic administration of blood products, even with abnormal CCT and TEG R-time and MA, 1 patient experienced bleeding (0.7%)[43]. Also, a recent study in 90 patients with cirrhosis undergoing central venous cannulation demonstrated that a prolonged TEG K-time (≥ 3.05 min) could not predict bleeding complications (accuracy 69.4%, P = 0.047)[44]. These studies indicate that post-procedural bleeding events are rare, implying that uncorrected coagulopathy does not modify the post-procedural outcome. Nevertheless, coagulation tests can be utilized to evaluate the severity of liver disease or the patient's baseline hemostatic function and to provide a baseline to guide management in the case of post-procedural bleeding.

Most of the latest guidelines recommend against using CCTs and correction of coagulopathy before undergoing common gastrointestinal procedures in patients with stable cirrhosis. Also, there are no recommendations for or against using TEG in such patient populations (Table 5)[15,23,45,46]. However, in patients with severely abnormal coagulation parameters or thrombocytopenia undergoing a moderate- to high-risk procedure, clinical judgment regarding prophylactic blood transfusion should consider the possible benefits and risks (Figure 5)[7,15].

Use of TEG in cirrhosis with active bleeding

Bleeding related to portal hypertension, variceal and non-variceal, is primarily managed with local measures such as endoscopic band ligation, laser or injection therapy, and by lowering portal pressure using vasoactive drugs than pro-hemostatic therapy. The observation that variceal bleeding in patients on anticoagulants was not severe or associated with worse outcomes compared to patients who are not on anticoagulants confirms that the role of the hemostatic system in variceal bleeding, if present, is minor[47]. Randomized controlled studies have shown that in cirrhotic patients with variceal and non-variceal bleeding, using VETs to guide blood product transfusion did not result in superior control of bleeding nor any morbidity or mortality benefit compared to CCTs[48-50]. However, the transfusion requirement was significantly lower in the VET group. Although the study by Kumar *et al*[51] demonstrated significantly shorter ICU stays using TEG-guided resuscitation, there was no difference in other outcomes. Nevertheless, it is questionable whether in active variceal bleeding, VETs-guided pro-hemostatic therapy is beneficial or contributes to the control of bleeding when the standard treatment with vasoactive drugs and endoscopic therapy is provided.

If local measures and portal pressure-lowering drugs cannot contain bleeding, the decision to correct coagulopathy by transfusing blood products should be considered on a case-by-case basis[13]. Since VETs are quicker and more accurate than CCTs and provide a more practical understanding of fibrinolysis, which may indicate the need to start antifibrinolytic therapy, they have a theoretical advantage over CCTs in guiding the management of active bleeding.

Unlike pressure-driven bleeding, AICF arises due to disturbed hemostatic mechanisms[15]. Antifibrinolytic therapy, such as epsilon aminocaproic acid or tranexamic acid, is potentially effective, inhibiting the fibrin clot's dissolution. Neither agent is thought to have inherent hypercoagulable risk, except in the case of a preexisting pathological thrombus such as PVT. The "native TEG" can detect this condition in liver disease patients by the presence of an increase in LY30[17].

TEG-based algorithms may allow targeted and specific blood product transfusions in patients with severe bleeding (*e.g.*, FFP or cryoprecipitates)[17]. However, the threshold values of various VETs to trigger transfusion are yet to be validated in appropriate clinical studies.

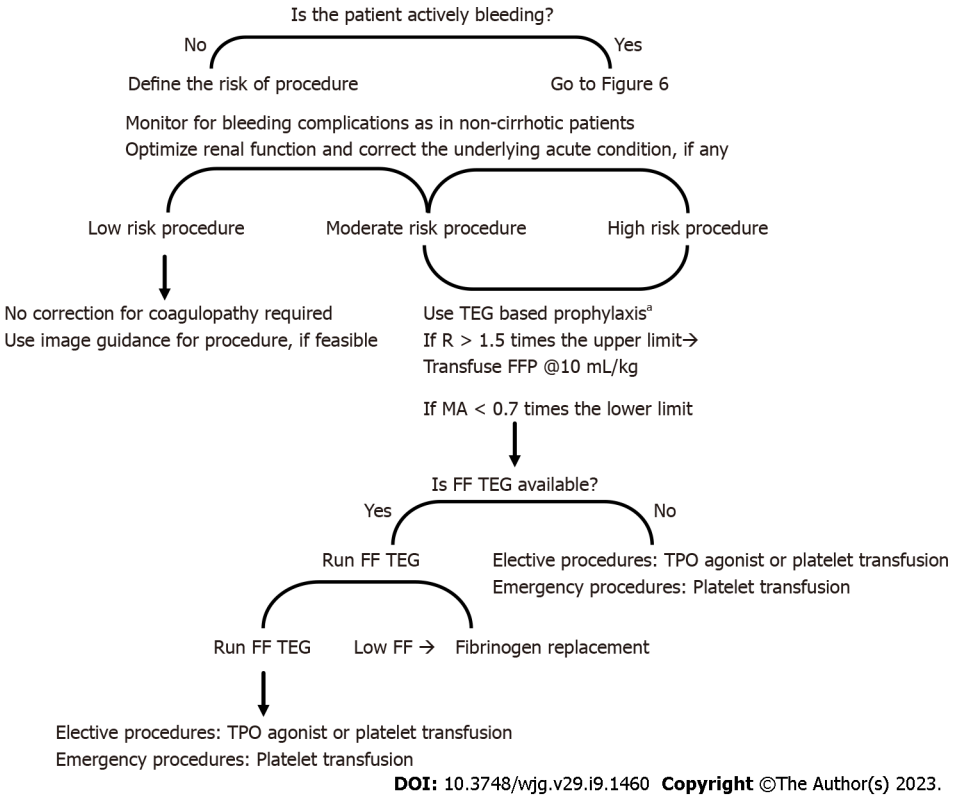
Heparin-like effect in cirrhosis

A stressful condition such as surgery or sepsis can trigger the release of endogenous glycosaminoglycans (GAGs) (*e.g.*, heparin sulfate and dermatan sulfate) from the endothelium glycocalyx layer or mast cell, which, when shed, retain their anticoagulant activity[52,53]. This is thought to be an adaptive reaction to maintain the patency of progressively procoagulant microvasculature through endogenous heparinization, thus preventing spontaneous thrombosis.

Table 5 Thresholds for coagulation parameters prior to high-risk procedures in patients with cirrhosis

Parameters	EASL 2022	ISTH 2021	AASLD 2021	AGA 2021
PT/INR	Against routine evaluation and correction	Against correction	Against correction	Against routine evaluation and correction[a]
Platelet count	Against correction[b]	Against correction[b]	Against correction	Against routine evaluation and correction[a]
Fibrinogen	Against routine correction	Against routine evaluation	Against correction	No specific recommendation
TEG	Against routine evaluation[c]	Do not use routinely	Do not use routinely	No specific recommendation

[a]In case of severe coagulopathy, prophylactic blood transfusions should be considered on case-to-case basis by evaluating potential benefits and risks in consultation with a hematologist.
[b]If the bleeding cannot be controlled by the local hemostasis method, administration of platelet concentrate or thrombopoietin receptor agonist can be considered if the platelet count is < 50000 × 10^6/L.
[c]May provide a baseline coagulation status and guide in the case of bleeding events.
AASLD: American Association for the Study of Liver Diseases; AGA: American Gastroenterological Association; EASL: European Association for the Study of the Liver; INR: International normalized ratio; ISTH: International Society on Thrombosis and Hemostasis; PT: Prothrombin time; TEG: Thromboelastography.



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Figure 5 Algorithm for coagulation factor administration in the cirrhotic patient with coagulopathy undergoing an invasive procedure. FF: Functional fibrinogen; FFP: Fresh frozen plasma; MA: Maximum amplitude; TEG: Thromboelastography; TPO: Thrombopoietin.

Endogenous GAGs may increase the bleeding risk in some patients. This was illustrated by Senzolo *et al*[54], where GAGs affected hemostasis in cirrhotic patients with sepsis. Another prospective analysis further confirmed the presence of an endogenous heparinoid in patients with cirrhosis and acute variceal bleeding and was found to be associated with bleeding-related mortality[55]. After appropriate therapy, endogenous heparinoids are cleared with normalization of the coagulation profile, emphasizing the association between the coagulation cascade and inflammatory pathways.

Although CCTs are insensitive to this effect, the native TEG is extremely sensitive to the presence of heparin and heparin-like substances, which is detectable by an increased R-time on TEG analysis[56]. Adding heparinase I, which cleaves heparin-like compounds, can demonstrate a heparin-like effect due to elevated GAGs, correlating with an anti-Xa activity[57]. Therefore, heparinase TEG will normalize the prolongation of the R-value observed with native TEG. Thus, TEG helps differentiate between a coagulation factor deficiency and heparin-produced coagulopathy by using heparinase-modified TEG

and the native TEG (Table 4).

TEG in orthotopic liver transplant

Kang *et al*[58] at the University of Pittsburgh introduced TEG-based algorithms to guide blood product transfusion for correcting coagulopathy in orthotopic liver transplantation in the early 1980s (Figure 6). It was shown that TEG reduced transfusion requirements by 33% compared with a historical cohort. Secondary endpoints like re-intervention for bleeding, AKI, or hemodynamic instability were significantly lower in the VET group. Although numerous studies have described the usefulness of VET in lowering transfusion requirements in liver transplant (LT), most of these studies commonly compared the results with historical cohorts having a relatively high baseline transfusion rate[59,60]. A recent study of 60 LT patients showed no significant differences with and without VET monitoring though overall transfusion was low, with many patients receiving no transfusion[61]. As bleeding and transfusion management continues to evolve, the results of these earlier studies cannot be easily employed in the present era. Also, the thresholds described for VET for initiating transfusion are still to be established, and values may be substantially above the normal ranges before an intervention is advised.

A significant proportion of patients undergoing LT will inevitably have enormous blood loss, and VET can be helpful in such occasions to enable goal-targeted treatment and assess the effectiveness of any therapeutic intervention. The short turnaround times of VET (10-20 min) are vital for directing therapy and averting inappropriate transfusion during surgery and in the ICU. Monitoring coagulation with functional fibrinogen TEG (Table 4) for goal-directed fibrinogen substitution seems more appropriate and avoids unnecessary platelet transfusions. This is particularly important in LT, as platelet administration is associated with a substantial decline in 1-year survival[62].

Fibrinolysis and orthotopic liver transplant

It is well known that increased fibrinolytic activity can occur at any juncture during LT. However, it is significantly enhanced during the anhepatic period due to a lack of tissue plasminogen activator (tPA) clearance[63]. Also, it may become most pronounced in the post-reperfusion stage by an erratic upsurge in tPA, leading to diffuse uncontrolled bleeding due to primary hyperfibrinolysis[64]. If the graft function is good, hyperfibrinolysis after reperfusion is usually self-limiting and does not require treatment. However, in the presence of an inadequately functioning graft, it may persist[65]. During LT, prophylactic antifibrinolytic agents were often used in earlier years because of the high mortality associated with tremendous blood loss, and the potential peril associated with antifibrinolytics was minor. As massive bleeding is currently less frequent, there is a preference towards the selective use of antifibrinolytics only in high-risk patients. Systemic fibrinolysis can be efficiently detected using VETs (demonstrated by increased or worsening LY30 and LY60), which may not be possible with CCTs. Thus, the transfusion requirement may be decreased with VET use in liver transplantation, where hyperfibrinolysis commonly occurs.

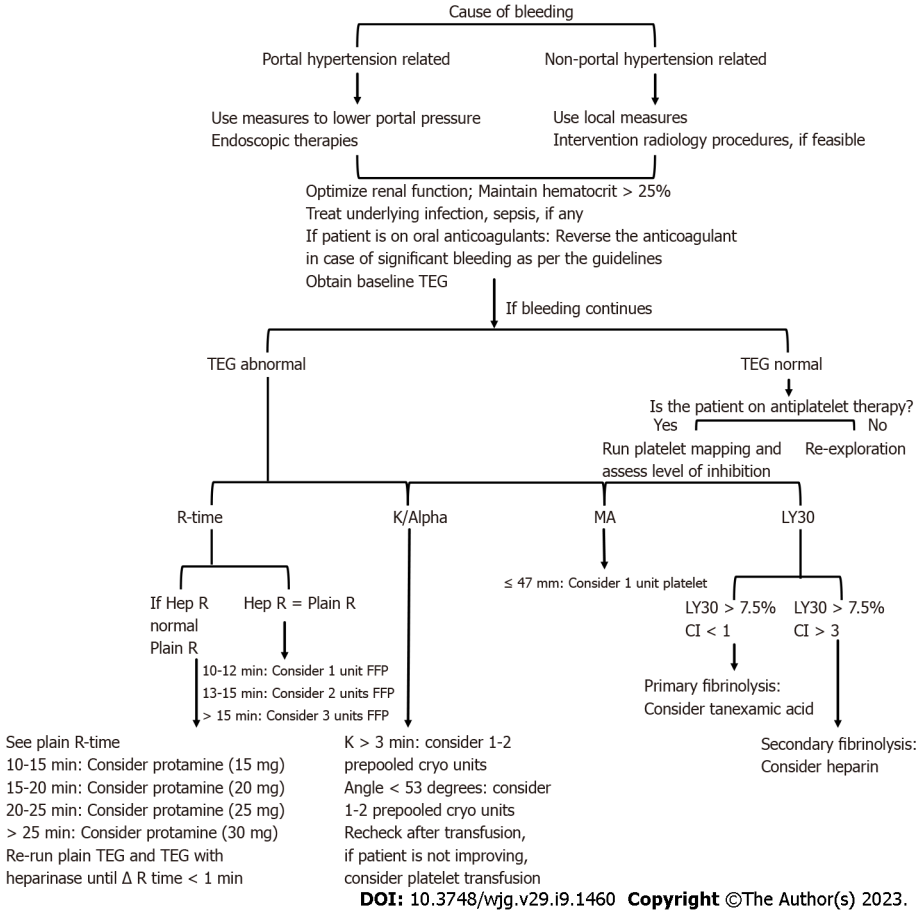
TEG and hypercoagulability

The risk of developing VTE is similar in cirrhotic and non-cirrhotic patients[15,23]. Hypercoagulability detected on TEG can either be due to shortened R or K, enhanced clot strength (MA), or a combination of both. Huang *et al*[66] observed a significantly shorter R in cirrhosis with non-malignancy PVT. Zanetto *et al*[67] found that elevated MA was associated with PVT in cirrhotic patients with hepatocellular carcinoma. Given that malignancy itself could also cause hypercoagulation, the clinical use of TEG in this setting may be questionable. In another study, hypercoagulability was defined as the presence of at least 2 of the following criteria: reduced R, reduced K, raised α, or increased MA. Hypercoagulability was not associated with PVT in cirrhosis[68].

In cirrhotic patients with elevated CCTs, we tend to avoid prophylactic anticoagulation in hospitalized patients. Presently, the European Association for the Study of the Liver Clinical Practice Guidelines in cirrhosis does not recommend using VETs to identify the risk of VTE[23]. Further prospective studies may explore the utility of TEG in predicting the risk of VTE during hospitalization.

Acute intracardiac thrombi and PEs are rare, although a well-recognized, potentially fatal complication of LT, associated with high mortality. Krzanicki *et al*[69] demonstrated that a hypercoagulable state is quite common during liver transplantation. A review of 27 case reports of TE in orthotopic LT showed that TEG indicated hypercoagulability in greater than 70% of cases[70]. Also, hypercoagulable TEG patterns correlated well with the formation of intracardiac thrombi. Indeed, a quick inspection of the rapid TEG after 5 or 10 min of clotting time might predict thrombosis, demonstrated by the increase in the MA. The clinical importance of hypercoagulability on TEG during LT is yet to be recognized. However, it would appear unreasonable to transfuse blood products or avoid anticoagulants based on raised CCTs when a hypercoagulable state is seen on TEG.

Patients with cirrhosis and VTE should be treated with anticoagulation, similar to other non-cirrhotic patients. In patients at increased risk of bleeding, unfractionated heparin (UFH) is the preferred anticoagulant, owing to its shorter half-life (45 min) and the availability of an effective antidote (protamine sulfate). aPTT is the most commonly used test to monitor UFH therapy. Although the anti-Xa activity



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Figure 6 Algorithm for guiding blood product transfusion by thromboelastography. Cryo: Cryoprecipitate; FFP: Fresh frozen plasma; Hep R: Heparinase R-Time; MA: Maximum amplitude; TEG: Thromboelastography.

assay is used explicitly for monitoring low molecular weight heparins, as they primarily inhibit factor Xa, it may also be superior to aPTT for titrating UFH[71].

Given that heparin activity mainly depends on the liver-derived activity of the heparin cofactor antithrombin III, monitoring heparin therapy with CCT in patients with cirrhosis is challenging. TEG may provide a better representation of the *in vivo* heparin effect than aPTT[72,73]. A higher concentration of heparin tends to be associated with larger R-values with dose-dependence. Levels of anti-factor Xa activity correlate with the R-value of TEG. In addition, TEG can help diagnose and treat heparin-induced coagulopathy. Thus, platelet and enzymatic hypercoagulability demonstrated with TEG mandates aggressive treatment with a direct thrombin inhibitor.

LIMITATIONS OF TEG

Like any other test, TEG is associated with certain limitations. It measures blood coagulation *in vitro* instead of during flow within the vasculature, and as such does not reflect the endothelium's function in coagulation. Inherently, the test is less sensitive to platelet adhesion and interactions between vWF and protein C and S system. TEG results do not correlate with the effects of hypothermia, as TEG is performed at 37 °C. Kaolin cannot effectively detect alterations in the extrinsic coagulation pathway, as it only activates the intrinsic coagulation pathway. Thus, INR is still the gold standard for monitoring warfarin therapy, and TEG may overlook a clinically significant coagulopathy. TEG detects fibrinolysis only when tPA levels are 5 times normal. Studies have shown that using plasmin-α2-antiplasmin as a biomarker for fibrinolysis can detect fibrinolytic activation in over 80% of severely injured patients, whereas TEG detected hyperfibrinolysis in only 5%-18%. Each TEG run generally takes 30 min to an hour, and only a few tests can run simultaneously, unlike CCT. The optimization of TEG is essential in providing appropriate patient laboratory testing. Additionally, testing should be performed by trained personnel and is susceptible to technical variations.

CONCLUSION

VETs are increasingly used as "point-of-care" tests, providing a real-time, dynamic picture of complex coagulation aberrations (*e.g.*, hypocoagulability, hypercoagulability and hyperfibrinolysis) in cirrhotic patients. In cirrhosis, all patients undergoing a high-risk invasive procedure or who are actively bleeding should undergo TEG at initial evaluation, if this testing is available. Any reasonable TEG-based strategy will likely represent an improvement over strategies using traditional coagulation tests. The best approach will be to use TEG supplemented by platelet count and fibrinogen measures. TEG is a promising diagnostic modality, but given the limited clinical trials, there are no consensus guidelines for its use. Further prospective studies are required to validate TEG algorithms for use in the context of patients with cirrhosis.

FOOTNOTES

Author contributions: Kataria S and Juneja D wrote the manuscript, researched the project, prepared the figures and tables, and performed data acquisition; Singh O reviewed draft and final versions of the manuscript.

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ORIGINAL ARTICLE

Basic Study

Adenosine 2A receptor contributes to the facilitation of post-infectious irritable bowel syndrome by γδ T cells *via* the PKA/CREB/NF-κB signaling pathway

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Abstract

BACKGROUND

Immunological dysfunction-induced low-grade inflammation is regarded as one of the predominant pathogenetic mechanisms in post-infectious irritable bowel syndrome (PI-IBS). γδ T cells play a crucial role in innate and adaptive immunity. Adenosine receptors expressed on the surface of γδ T cells participate in intestinal inflammation and immunity regulation.

AIM

To investigate the role of γδ T cell regulated by adenosine 2A receptor (A2AR) in PI-IBS.

METHODS

The PI-IBS mouse model has been established with *Trichinella spiralis (T. spiralis)* infection. The intestinal A2AR and A2AR in γδ T cells were detected by immunohistochemistry, and the inflammatory cytokines were measured by western blot. The role of A2AR on the isolated γδ T cells, including proliferation, apoptosis, and cytokine production, were evaluated *in vitro*. Their A2AR expression was measured by western blot and reverse transcription polymerase chain reaction (RT-PCR). The animals were administered with A2AR agonist, or A2AR antagonist. Besides, γδ T cells were also injected back into the animals, and the

parameters described above were examined, as well as the clinical features. Furthermore, the A2AR-associated signaling pathway molecules were assessed by western blot and RT-PCR.

RESULTS

PI-IBS mice exhibited elevated ATP content and A2AR expression ($P < 0.05$), and suppression of A2AR enhanced PI-IBS clinical characteristics, indicated by the abdominal withdrawal reflex and colon transportation test. PI-IBS was associated with an increase in intestinal T cells, and cytokine levels of interleukin-1 (IL-1), IL-6, IL-17A, and interferon-α (IFN-α). Also, γδ T cells expressed A2AR *in vitro* and generated IL-1, IL-6, IL-17A, and IFN-α, which can be controlled by A2AR agonist and antagonist. Mechanistic studies demonstrated that the A2AR antagonist improved the function of γδ T cells through the PKA/CREB/NF-κB signaling pathway.

CONCLUSION

Our results revealed that A2AR contributes to the facilitation of PI-IBS by regulating the function of γδ T cells *via* the PKA/CREB/NF-κB signaling pathway.

Key Words: Irritable bowel syndrome; Adenosine 2A receptor; γδ T cells; Post-infectious irritable bowel syndrome; Signaling pathway; Regulation

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Core Tip: Immunological dysfunction-induced low-grade inflammation is regarded as one of the most important pathogenetic mechanisms in post-infectious irritable bowel syndrome. γδ T cells play a crucial role in innate and adaptive immunity. The adenosine molecule and receptors regulate intestinal inflammation and immunity. Through the PKA/CREB/NF-κB signaling pathway, we showed that adenosine 2A receptor contributes to the facilitation of post-infectious irritable bowel syndrome by T cells.

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INTRODUCTION

Irritable bowel syndrome (IBS), a functional gastrointestinal disorder with abnormal bowel habits and/or faecal traits, has been increasing in prevalence in developing countries since 1990[1]. The concept of post-infectious IBS (PI-IBS) was derived from clinical observations of patients with previous acute gastrointestinal infections[2]. Patients often suffer from refractory discomfort, low quality of life, and high healthcare economic burden, but current treatments reap unsatisfactory therapeutic results due to the lack of specific targets[3]. Numerous studies in the literature have shown that 1/3 of IBS patients have a history of gastrointestinal infection, suggesting that a persistent inflammatory response may explain the inconsistency between the intestinal response of PI-IBS patients and the normal gut[4]. Therefore, researchers generally agree that PI-IBS is also essentially a post-inflammatory immune dysfunction[5] and that reducing the intestinal inflammatory response is expected to improve clinical symptoms and life quality. For the potential therapeutic target of PI-IBS, upregulation of EphA2 expression and activation of the NF-κB signaling pathway have been reported to alleviate PI-IBS-related symptoms[6], while miRNA-510 has also been proved to regulate the intestinal inflammatory response in PI-IBS by targeting PRDX1[7].

γδ T cells are a subpopulation of T lymphocytes with a distinct phenotype and function, although they represent only a small proportion of the total T cells. γδ T cells bridge the gap between innate and adaptive immunity, determining the type of immune response and maintaining homeostasis in mucosal tissues. In inflammatory and immune responses, γδ T cells play a complex dual role depending on the context. On the one hand, γδ T cells present antigens to immune cells and trigger the recognition of these antigens by immune cells, leading to an adaptive immune response, promoting the secretion of chemokines and cytokines, and recruiting inflammatory cells[8,9]. On the other hand, γδ T cells inhibit the inflammatory process by conducting regulatory cytokines such as interferon-α (IFN-α), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and IL-17 and eliminating over-activated inflammatory effector cells such as macrophages and polymorphonuclear leukocytes through cytotoxic effects[10]. In animal models of experimental autoimmune encephalomyelitis, rheumatoid arthritis and non-fatty

diabetes, γδ T cells are proved to be the causative factor whereas rats with colitis and sepsis and mice with EAU were found to benefit from their presence[11]. According to our previous research, we have demonstrated that γδ T cells could alleviate PI-IBS through promoting Th17 polarization *via* their HSP70 receptor[12].

One of the many receptors expressed on the surface of γδ T cells is adenosine 2A receptor (A2AR), a classical G protein-coupled receptor with a high affinity for adenosine (ADO)[13]. A2AR is expressed in many immune cells, such as regulatory T cells, cytotoxic T cells, macrophages, *etc*. Its homologous family members include four isoforms, namely A1R, A2aR, A2bR, and A3R. These ARs are distributed in different tissues. For example, A1R is in the small intestine, A2aR and A2bR are in the cecum and colon, and A3R is in the ileum and colon[14]. A2AR forms the CD39-CD73-A2AR adenosine metabolic pathway with CD39 and CD73: first, CD39 cleaves extracellular ATP into AMP, which then binds to CD73 and is converted to ADO by CD73. ADO then binds to A2AR, which in turn interacts with Gs family proteins to increase intracellular cAMP levels and activate the downstream PKA/CREB-related pathway. Thus, the adenosine-A2AR pathway plays an important role in protecting normal organs and tissues from the autoimmune response of immune cells[15-17]. There are fewer reports on A2AR and intestinal inflammatory injury. Researchers have found that ADO can inhibit intestinal inflammatory injury in chronically septic mice *via* interacting with A1AR and A2AR[18]. And according to the research from Hou *et al*[19], electroacupuncture reduced visceral pain in rats with inflammatory bowel disease by interfering with adenosine receptors. However, there are no studies correlating A2AR with the pathogenesis of IBS, especially PI-IBS. Recently, Sun *et al*[20] reported that A2AR is able to bind to mGluR5 and exhibits a unique role in maintaining the function of the intestinal barrier in enteric glial cells against hypoxia-induced cellular damage. The above findings suggest that ARs, especially the A2AR isoform, may play a key role in the pathogenesis of PI-IBS and could potentially be a novel target for intervention in PI-IBS progression.

Hence, considering that the correlation between A2AR and PI-IBS remains unclear, the function of A2AR on the surface of γδ T cells has not been fully elucidated, and the precise A2AR-related signaling pathway mediating PI-IBS progression in γδ T cells awaits to be explored. It is an urgent issue to evaluate the role of A2AR in regulating γδ T cells on PI-IBS pathogenesis.

MATERIALS AND METHODS

Animals and experimental design

Ninety-six female 57BL/6 mice were provided by the Medical Animal Center of the Hainan Medical College. All animals were housed in a sterile, suitable environment and fed *ad libitum* with food and water that met cleanliness standards. The animals used in the experiments were cared for and handled by the Chinese Guide for Laboratory Animals. The experimental procedures were examined and approved by the Animal Care and Utilization Committee of Hainan.

Animals were randomly divided into 4 groups (n = 24 per group): The control group, the PI-IBS group, the IBS + SCH58261 group, and the IBS + CGS21680 group. A quarter of mice were killed in each group, then intestinal A2AR protein and mRNA levels, cytokine expression and secretion plus signaling pathway activation were investigated, respectively. Another six mice were used for γδ T cell isolation. The remaining mice were used for A2AR lentivirus infection and cell reinfusion assay.

PI-IBS Model

The Trichinella spiralis (*T. spiralis*) infected the mice as described earlier in Lanzhou Animal Medical Institute[21,22]. Briefly, parasite larvae were isolated from cysts of SD rats, which have been infected with *T. spiralis* for 60 d and digested with 1.5% pepsin. Mice were then fed with larvae, which were resuspended in 0.2 mL of saline (300 larvae per mouse). At the same time, the control group was only fed with 0.9% saline.

Histopathological analysis

After 8 wk of infection, animals were euthanized, meanwhile, their ileal tissues were fixed with 10% formalin at 4 °C for 10 d, then dried in ethanol, and embedded in paraffin. The 5 micrometers sections were made. After dewaxing and hydration, they were stained with hematoxylin-eosin (HE). In addition, HE staining was used to assess inflammation scores according to a previous scoring system[23].

Abdominal withdrawal reflex

The abdominal withdrawal reflex (AWR) was administered to assess visceral hypersensitivity[24]. A catheter and air chamber were introduced *via* the anus of sedated animals. The air chamber was inflated to a capacity of 0.25/0.35/0.5/0.66 mL in 15 min on a number of occasions. The animals were allowed 30 s of rest between each distending period. The AWR criterion for scoring: When stimulated, the animals' mood is stable, 0 points; if they are unstable, occasionally bending their necks, 1 point; slightly contracting their abdomen and back muscles, 2 points; intensively contracting their abdomen muscles

and lifting the abdomen from the ground, 3 points; intensively contracting abdomen muscles, bowing abdomen, and lifting abdomen and perineum, 4 points.

Colon transportation test and stool scale

The colon transit test (CTT) was used to assess intestinal motility. After ingesting 0.4 mL of active carbon, the timing was documented. The entire feces collected during 8 h were graded using the Bristol stool scale: 1 point for normal-shaped stools; 2 points for stools that are soft or malformed; 3 points for stools that are watery[25].

Immunohistochemistry

Immunohistochemistry was performed on the cells as previously described[26]. Briefly, cells were fixed in suspension with 4% PFA for uniform distribution on the slides. After rinsing in phosphate-buffered saline (PBS), slides were incubated with anti-mouse A2AR monoclonal antibody (1:150; Abcam, Cambridge, United Kingdom) overnight at 4 °C and then with HRP secondary antibody (SP-9001, Beijing Sequoia Jinqiao, Beijing, China) for 1 h at room temperature. Reactivity was detected using the DAB reagent (Beijing Sequoia Jinqiao, Beijing, China). The primary antibody was substituted with PBS as a negative control. As the cell membrane was the main localization site of A2AR, the expression of A2AR was evaluated by a semi-quantitative integration technique. A2AR protein expression staining intensity was rated as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of positively stained cells in the visual field was categorized as 0 (5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), or 4 (> 75%). The evaluation was represented as the product of the positive rate score and staining intensity score, with a score of greater than or equal to 6 indicating a favorable evaluation. The above staining was examined using Nikon DR-Si2 cell counting software and digital image analysis was employed. Moreover, the scoring was done in a double-blind way, which was confirmed by two pathologists who were not involved in this study and unaware of the clinical-pathological data.

Western blotting

Western blot was used to detect altered protein expression. Ileal tissue samples were lysed with RIPA coupled with brief ultrasonic pulverization. After lysis was completed, the homogenate was centrifuged at 4 °C for 30 min, the precipitate was discarded and the supernatant was retained. The protein content of the supernatant was determined by the Bradford method. 40 μg of protein was taken for SDS-PAGE gel electrophoresis, and the protein blot was transferred to the PVDF membrane. Transfer conditions: β-actin, A2AR (200 mA, 90 min); PKA, p-PKA (200 mA, 120 min followed by 300 mA, 30 min); CREB, p-CREB (200 mA, 20 min followed by 300 mA, 15 min); NF-κB (200 mA, 120 min); claudin-1 (200 mA, 70 min), occludin (200 mA, 120 min); ZO-1 (200 mA, 120 min followed by 300 mA, 60 min); IL-1β (200 mA, 50 min); IL-6 (200 mA, 70 min); IL-17A (200 mA, 50 min); IFN-α (200 mA, 60 min). The primary antibodies presented in Table 1 were rabbit anti-mouse multi-clonal.

The diluted primary antibodies were incubated with the PVDF membrane at 4 °C for over 12 h. On the following day, the membrane was rinsed with TBST for three times, and ECL chemiluminescent test was performed. In a dark setting, the grayscale value was identified by improved chemiluminescence. The protein/actin grayscale value represented the relative protein expression level.

Real-time quantitative polymerase chain reaction

mRNA alteration was determined through Real-time quantitative polymerase chain reaction (PCR). Briefly, RNA was extracted from the tissue of the terminal ileum by using Trizol and DNase I. Primers were constructed based on the gene sequences of mice (Table 2). β-Actin served as an internal reference. The RNA was then reverse transcribed to cDNA according to the steps of the TAKARA PrimeScript kit (TAKARA-RR047A), and real-time quantitative PCR was performed according to the following procedure: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 1 min; 30 cycles of amplification and qualification at 57 °C and 60 °C; the ultimate step was prolongation at 72 °C for 7 min. Results are expressed as the ratio of target genes to control genes.

Measurement of ATP concentration

The tissue samples were sonicated and centrifuged at 4 °C for 15 min followed by the addition of double distilled water (1:9). The 10% homogenate was continued to be centrifuged at 3000 rpm for another 10 min. The supernatant was collected for subsequent analysis.

The ATP concentration was determined by the colorimetric method. Specifically, the OD value of the liquid in the colorimetric tube was measured at 636 nm. ATP concentration was calculated as: ATP concentration (mol/g prot) = (measured OD - control OD)/(standard OD - blank OD) × standard concentration (1000 mol/L)/sample concentration (g prot/L).

Isolation and purification of γδ T cells

γδ T cells were extracted from the spleen of animals[27]. Spleens were collected aseptically from 8 to 10-week female mice and stored in 1 × PBS on ice. Splenocytes were then isolated and red blood cells were

Table 1 The dilution ratio for primary antibodies

Primary antibodies	Dilution ratio
β-actin	1:1000
A2AR	1:500
NF-κB	1:2000
PKA	1:1000
p-PKA	1:2000
CREB	1:1000
p-CREB	1:1000
ZO-1	1:1000
Occludin	1:1000
Claudin-1	1:1000
IL-1β	1:1000
IL-6	1:1000
IL-17A	1:500
IFN-α	1:1000

A2AR: Adenosine 2A receptor; IL: Interleukin; IFN-α: Interferon-α.

Table 2 Primers used in this study

Name	Primer	Sequence	Size
β-actin	Forward	5′-CACGATGGAGGGGCCGGACTCATC-3′	240 bp
	Reverse	5′-TAAAGACCTCTATGCCAACACAGT-3′	
NF-κB	Forward	5′-CACCGGATTGAAGAGAAGCG-3′	194 bp
	Reverse	5′-AAGTTGATGGTGCTGAGGGA-3′	
CREB	Forward	5′-GCTGGCTAACAATGGTACGG-3′	230 bp
	Reverse	5′-CCATAACAACTCCAGGGGCA-3′	
PKA	Forward	5′-GGGCGTGCTGATCTATGAGA -3′	169 bp
	Reverse	5′-TCGCTTTGTCAGATCCACCT-3′	
A2AR	Forward	5′-GCCTCTTCTTCGCCTGCTTTGTCC-3′	140 bp
	Reverse	5′-GCCCTTCGCCCTCATACCCGTCAC-3′	

lysed with a hypotonic solution. γδ TCR cells were selected by depletion of CD11b, B220, CD4 and CD8 cells, negative selection using biotinylated antibodies and EasySep Biotin Positive Selection Kit (StemCell) according to the manufacturer's protocol.

The magnetic separation process was carried out as follows: Biotin Anti-CD11b, Biotin Anti-B220, Biotin Anti-CD4, and Biotin Anti-CD8 (1/100 × dilution, 10 μL/mL) were added respectively, then the whole system was mixed thoroughly and incubated at room temperature for 15 min. After that, 10 mL 1 × PBS + 0.526 mmol/L EDTA + 2% FBS was added and rotated at 1.4 k for 5 min at 4 °C. Discarding the supernatant and then the cells were resuspended at 1 × 10^8 cells/mL in the mentioned buffer. Then, Biotin Select Cocktail (100 μL/mL) was added and mixed thoroughly, following the incubation at room temperature for 15 min. Next, the beads from the EasySep kit were vortexed for 15 to 30 min and added (50 μL/mL), the new system was mixed thoroughly and incubated at room temperature for 10 min. At room temperature, tubes were placed into silver EasySep magnets, incubated for 5 min, and the supernatant fraction was poured into a new 15 mL tube. Then the cells were counted for further cell sorting.

The antibodies for sorting were as follows: γδTCR Alexa 488 (1/100 × dilution), CD8 PE (1/100 × dilution), CD4 PerCP Cy5.5 (1/100 × dilution), CD11b APC (1/100 × dilution) and B220 PEcy7 (1/100 × dilution). Before sorting, cells were stained at 4 °C for 30 min on ice with the mentioned antibodies.

Then, we washed the cells with 1 × PBS and resuspended to 5 × 10^6 cells/mL for sorting[28].

Cell proliferation assays

The proliferation of isolated γδ T cells was detected by CCK8 assay. Cells were resuspended in 100 μL of the medium at the density of 5 × 10^4 cells per well, then added to 96-well plates and waited for the cells to stick. After that, 100 nmol/L CGS-21680 or 1 μnmol/L SCH58261 was added to different wells of the 96-well plate, and the cells were continued to be cultured at 37 °C with 5% CO_2 for more than half an hour. Finally, 20 μL CCK8 was added to each well and the incubation was continued at 37 °C, 5% CO_2 for another 2 h, and the OD value at 450 nm was measured by Multiscan Spectrum.

Cell apoptosis assays

The extracted γδ T cells were uniformly distributed in six-well plates (5 × 10^5 cells/well). Subsequently, 100 nnmol/L CGS-21680 or 1 μnmol/L SCH58261 were added, respectively, and incubated at 37 °C with 5% CO_2 for 0.5 h. After that, the apoptosis rate was detected by fluorescence-activated cell sorting (FACS).

Cytokines production

ELISA was performed to determine the levels of IL-1β, IL-6, IL-17A, and IFN-α in cultivated γδ T cells and colonic tissue supernatants. Specifically, the original supernatant was removed and replaced with a new medium (RPMI-1640), and typical culture conditions were re-established for another 24 h. The content of cytokine proteins in the supernatant was quantitatively analyzed by using IL-1, IL-6, IL-17A, and IFN-α high sensitivity (0.25-16 pg/mL sensitivity range) ELISA kits (R&D Systems, Minneapolis, Minnesota, United States) according to the manufacturer's instructions.

Cell reinfusion

Isolated γδ T cells were treated with SCH58261 or CGS21680 at 37 °C, 5% CO_2 for 48 h. The cell concentration was then adjusted to 2 × 10^6 cells/mL with RMPI-1640. Mice were injected separately with γδ T cells at an inoculum of 2 × 10^6 cells per mouse. Clinical characteristics were observed as described previously.

Transient transfection

LV-mA2AR-shRNA was synthesized by Fubio (Suzhou) Biopharmaceutical Technology Co., Ltd. and Suzhou Genepharma Co., Ltd. The sequence is as follows: the sense is 5'-GGAGACAGCUGA-AGCAGAUTT-3'; the antisense sequence is 5'- AUCUGCUUCAGCUCUCUCCTT-3'. The HEK293T A2AR knock-down cell line was obtained by transfecting LV-mA2AR-shRNA with γδ T cells and incubating at 37 °C for 48 h after transfection. Similarly, the HEK293T cell line infected with a negative control sequence (sh-NC) was constructed.

Statistical analysis

Data are represented as mean ± SD. All data were analyzed using Grubb's test, followed by one-way analysis of variance (ANOVA) and Levene's test to assess the homogeneity of variance, and finally Ducan's multiple comparison test for multiple comparisons (SPSS 22.0 software). $P < 0.05$ were considered statistically significant.

RESULTS

The establishment of PI-IBS mouse model and the impact of A2AR on PI-IBS

First, we chose the *T. spiralis* infection to construct the PI-IBS mouse model. On day 56 after the *T. spiralis* infection, the model was evaluated, which demonstrated that there was no obvious inflammation in the animal colon (Figure 1), but the AWR rating was significantly higher than that of the control group, and the results of CTT experiment were significantly abnormal, as shown by the shortened first black stool time and a higher Bristol stool level (Tables 3 and 4). This indicated that a successful PI-IBS mouse model has been established.

After that, we applied the A2AR agonist or antagonist to PI-IBS mice in order to find potential interventions for curing PI-IBS. A further novel finding was that only the injection of A2AR antagonist SCH58261 significantly relieved the severe clinical manifestations of the model animal whereas A2AR agonist CGS21680 could not. At this stage of understanding, these findings suggest that A2AR may have a crucial impact on the development of PI-IBS.

The intestinal levels of ATP and A2AR expression are upregulated in PI-IBS mice

To further assess the relationship between A2AR and PI-IBS, we compared the altered expression of A2AR and ATP content in mice with PI-IBS or in normal mice, as A2AR plays a vital role in the conversion of ATP. From the results, it is clear that compared with the control group, intestinal ATP

Table 3 The effect of adenosine 2A receptor on the abdominal withdrawal reflex score in post-infectious irritable bowel syndrome mouse

Distending air volume (mL)	AWR			
	0.25	0.35	0.5	0.65
Control (n = 6)	0.00 ± 0.00	1.67 ± 0.05	2.52 ± 0.18	3.65 ± 0.08
PI-IBS (n = 6)	0.00 ± 0.00	2.13 ± 0.12[a]	3.15 ± 0.14	3.90 ± 0.15
IBS + SCH58261 (n = 6)	0.00 ± 0.00	1.79 ± 0.12[c]	2.90 ± 0.25	3.57 ± 0.05
IBS + CGS21680 (n = 6)	0.00 ± 0.00	2.01 ± 0.42	3.09 ± 0.53	3.62 ± 0.11

[a]P < 0.05 *vs* the control group.
[c]P < 0.05 *vs* the PI-IBS group.
AWR: Abdominal withdrawal reflex; PI-IBS: Post-infectious irritable bowel syndrome.

Table 4 The effect of adenosine 2A receptor on the intestinal mobility in post-infectious irritable bowel syndrome mouse

Group	First black stool time (min)	Bristol stool grade
Control (n = 6)	407 ± 10.33	1.00 ± 0.00
PI-IBS (n = 6)	132 ± 12.59[a]	2.67 ± 0.52[a]
IBS + SCH58261 (n = 6)	246 ± 14.20[c]	1.33 ± 0.51[c]
IBS + CGS21680 (n = 6)	168 ± 11.38	2.66 ± 0.54

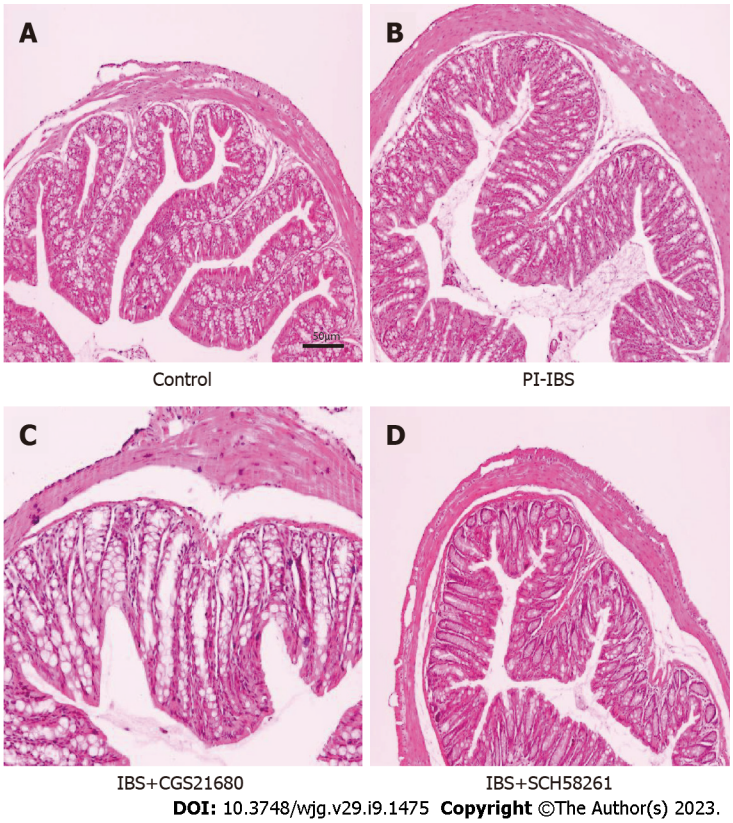
[a]P < 0.05 *vs* the control group.
[c]P < 0.05 *vs* the PI-IBS group.
PI-IBS: Post-infectious irritable bowel syndrome.

levels in PI-IBS mice rose considerably (P < 0.01, Figure 2A) as did A2AR protein expression (P < 0.01, Figure 2B-D). Intestinal ATP levels and A2AR expression were further enhanced when the PI-IBS mice were treated with the A2AR agonist CGS21680 (P < 0.05, Figure 2A-D). At the same time, the levels of inflammatory factors such as IL-1, IL-6, IL-17, and IFN-α were also further boosted compared to the PI-IBS model group (Figure 2E-G). Interestingly, when SCH58261, an antagonist of A2AR, was administered in PI-IBS mice, the significant increase in ATP levels and the up-regulation of A2AR expression triggered by PI-IBS was reversed (Figure 2A-D), followed by the drop of inflammatory factors' expression compared with the PI-IBS group, including IL-1, IL-6, IL-17 and IFN-α (Figure 2E-G).

Intestinal epithelial tight junctions (TJs) proteins, such as ZO-1, Occludin, and Claudin-1, play a vital role in maintaining the epithelial barrier function to restrict the paracellular movement of harmful substances across intestinal mucosa[29]. The disrup- tion of the TJs barrier could increase dysregulated immune reactions, such as the activation of mucosal immune response and the permeation of noxious molecules, and thus inducing gut inflammation[30,31]. Thus, we next tested the change of ZO-1, occludin, and claudin-1 expression. As we can see, the expression of TJ proteins ZO-1, Occludin, and Claudin-1 was sharply reduced in PI-IBS mice compared to the control, while agonism of A2AR further inhibited the expression of the proteins mentioned above. In line with the results in Figure 2A-D, treatment with the antagonism of A2AR SCH58261 also reversed the decreased expression of ZO-1, Occludin, and Claudin-1 in the PI-IBS group (Figure 2H-I). The results of the experiment found clear support for the hypothesis that A2AR is crucial to the pathophysiology of PI-IBS.

The upregulated A2AR in γδ T cells attributes to PI-IBS progression

It has been previously reported that the intestinal γδ T cells could exert an important role in a PI-IBS mouse model[32]. However, we wanted to further corroborate whether it is the altered A2AR expression in γδ T cells that directly influences the pathogenesis of PI-IBS. Before starting the exploration, we first purified γδ T cells. Figure 3A-C depicts the extraction and purification of γδ T cells from the spleen of PI-IBS mice for subsequent *in vitro* functional assessment through FACS sorting. Presently, we examined the A2AR expression in unpurified γδ T cells (the control group) and purified γδ T cells (the γδ T cell group) by immunohistochemical staining, respectively. The results were presented in Figure 3D and E. Notably, the A2AR expression level of γδ T cells was significantly higher than that in the control group, suggesting that A2AR was mainly highly expressed in γδ T cells. Furthermore, we treated γδ T cells with A2AR agonist CGS21680 and A2AR antagonist SCH58261, respectively, and we can visualize through Figure 3F and G that the A2AR expression level was further



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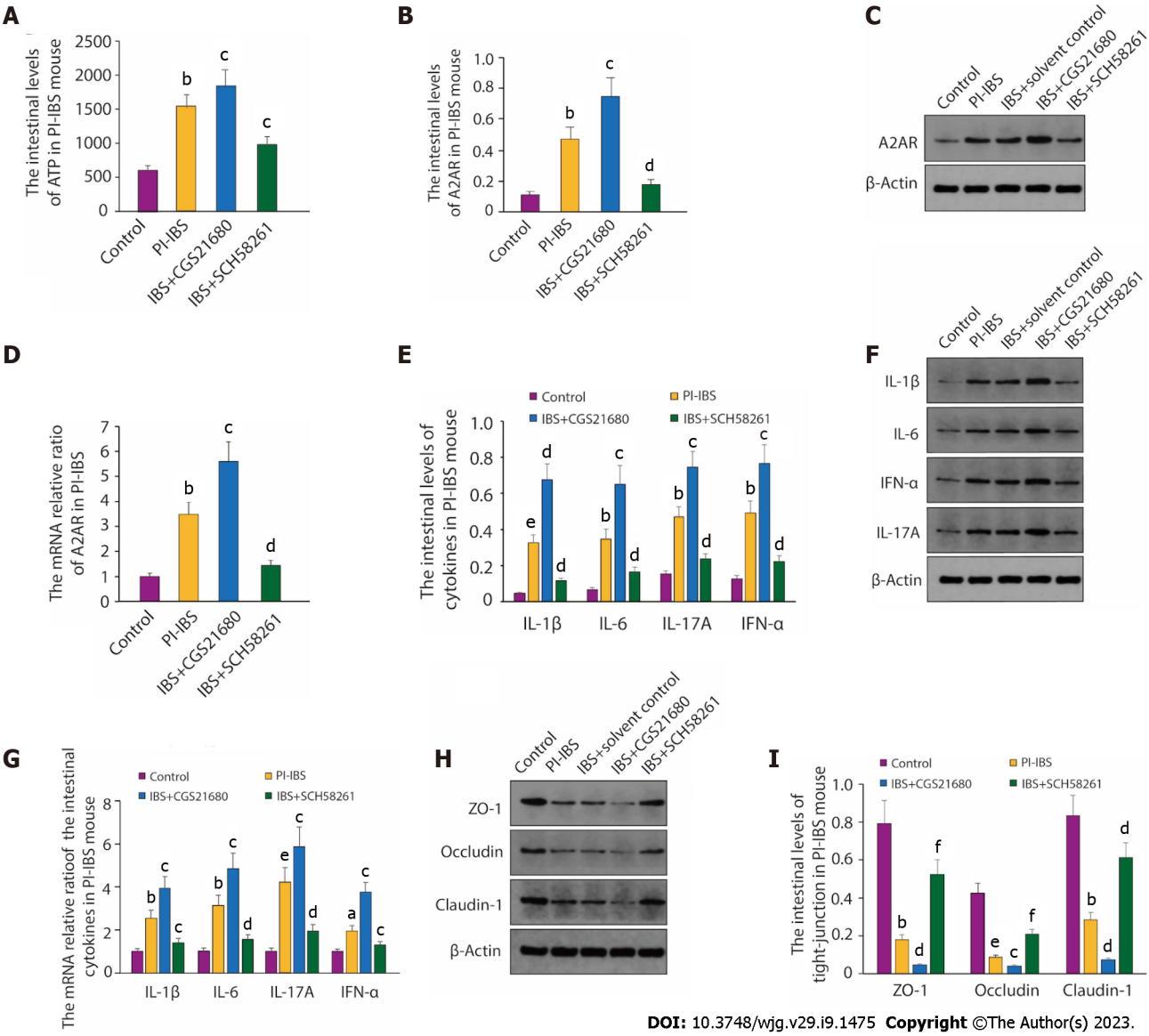
Figure 1 The effect of adenosine 2A receptor on the histopathological changes in post-infectious irritable bowel syndrome mouse. A and B: The Post-infectious irritable bowel syndrome (PI-IBS) model (B) exhibits no substantial inflammatory alterations in its colon tissue compared to the control animal (A); C: Adenosine 2A receptor (A2AR) agonist CGS21680 leads to some inflammatory alterations in the colon tissue of the PI-IBS model mice, including the infiltration of certain inflammatory cells; D: A2AR antagonist SCH58261 relieves inflammation in the colon tissue of the PI-IBS model mice. A2AR: Adenosine 2A receptor; PI-IBS: Post-infectious irritable bowel syndrome. Scale bars, 50 μm.

enhanced in T cells treated with A2AR agonists, while the A2AR expression in T cells was significantly downregulated after the administration of SCH58261 to inhibit A2AR.

Besides, western blot and reverse transcription PCR results further confirmed the immunohistochemical findings: the agonist significantly increased both A2AR protein and mRNA level in γδ T cells, whereas the antagonist did the opposite (Figure 3H-J). Taken together, these results revealed that γδ T cells were the main immune cell subtype mediating the upregulation of A2AR expression in the intestinal immune microenvironment. By interfering with A2AR expression within γδ T cells, the function of γδ T cells might be affected, which could in turn interfere with disease progression throughout PI-IBS.

The viability and function maintenance of γδ T cells is closely related to A2AR

To verify the conjecture of the previous chapter, we would like to further unveil the effect of A2AR on γδ T-cells' viability and function. First, we assessed the correlation between A2AR and γδ T-cell viability by the measurement of altered ATP content in γδ T cells and the percentage of proliferating γδ T cells. The results are shown in Figure 4A and B. When we treated γδ T cells with the A2AR agonist CGS21680, both the intracellular ATP content and the percentage of proliferating cells increased compared with the control γδ T cells, suggesting that A2AR activation could enhance the cell viability of γδ T cells; whereas the intracellular ATP content and proliferating percentage both decreased when the antagonist of cellular A2AR, SCH58261, was given. Subsequently, we further evaluated whether the apoptosis of γδ T cells was affected by A2AR, and the results demonstrated that inhibition of A2AR promoted the apoptosis of γδ T cells, while activation of A2AR greatly inhibited the apoptosis process (Figure 4C and D). Finally, we examined the function of γδ T cells. To be specific, we reinfused γδ T cells that have been treated differently into PI-IBS mice. A similar pattern of results was obtained in the expression of inflammatory factors, including IL-1β, IL-6, IL-17A and IFN-α in γδ T cells. Virtually, the addition of A2AR agonist CGS21680 promoted the expression of IL-1β, IL-6, IL-17A and IFN-α, while A2AR antagonist SCH58261 inhibited the expression of the above-mentioned inflammatory factors ($P < 0.05$, Figure 4E). The above results suggest that A2AR has an important effect on the viability and function of γδ T cells, especially on the secretion of inflammatory factors.



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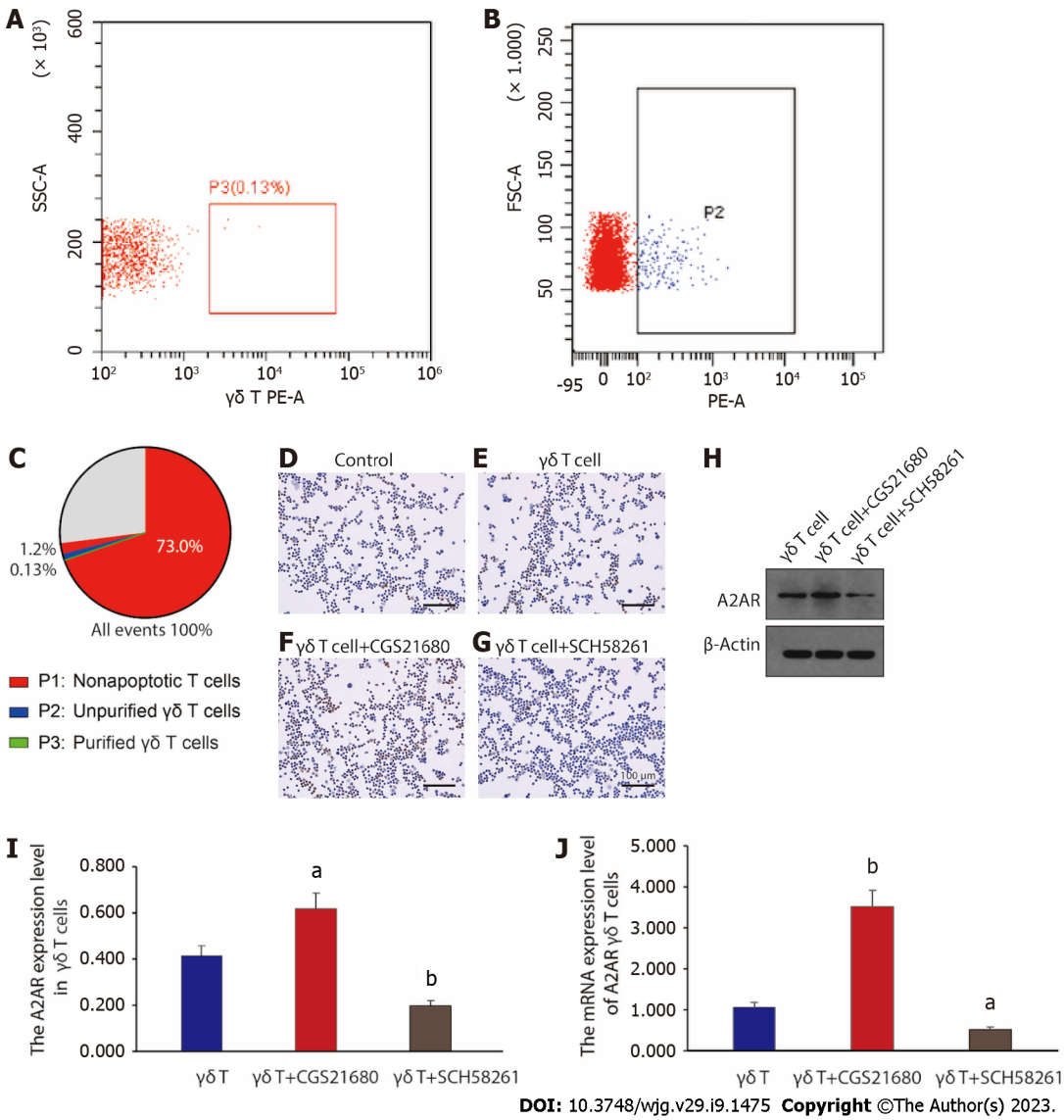
Figure 2 The intestinal ATP and adenosine 2A receptor expression in post-infectious irritable bowel syndrome mouse. A: The level of ATP in post-infectious irritable bowel syndrome (PI-IBS) mouse. The results were independently repeated three times; B and C: The intestinal levels of adenosine 2A receptor (A2AR) in PI-IBS mouse; D: The mRNA relative ratio of A2AR in PI-IBS mouse. The results were independently repeated three times; E and F: The intestinal levels of the inflammatory cytokines in PI-IBS mice were measured by ELISA and western blot, respectively. The ELISA results were obtained from six mice; G: The mRNA relative ratio of the intestinal cytokines in PI-IBS mouse. The samples were from mice mentioned in E and F. The results were independently repeated three times; H and I: The intestinal levels of tight junction proteins in PI-IBS mice were tested through western blot and quantified by image J software. β-Actin is used as the loading control for both western blot and reverse transcription polymerase chain reaction. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the Control group; $^{c}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* the PI-IBS group.

The regulation of A2AR on γδ T cells relies on the PKA/CREB/NF-κB signaling pathway

As ADO binds to A2AR, A2AR then interacts with Gs family proteins to increase intracellular cAMP levels and activate downstream PKA/CREB-related pathways[33], we next investigated the activation of signaling pathways in γδ T cells by western blot. The change of PKA/p-PKA, CREB/p-CREB, NF-κB/p-NF-κB protein level, and quantitative results elucidated that compared to control γδ T cells, treatment of γδ T cells with the A2AR antagonist SCH58261 significantly decreased the expression of PKA/CREB/NF-κB and reduced the phosphorylation levels of the above proteins; whereas the introduction of A2AR agonist CGS21680 further promoted the protein expression of PKA/CREB/NF-κB as well as the phosphorylation activation level of the above proteins (Figure 5A-D). Therefore, given the above results, we can safely draw the conclusion that the PKA/CREB/NF-κB pathway is the key downstream signaling pathway for γδ T cells to regulate the relevant clinical manifestations *via* A2AR during PI-IBS disease progression.

A2AR knockdown inhibits the inflammatory response

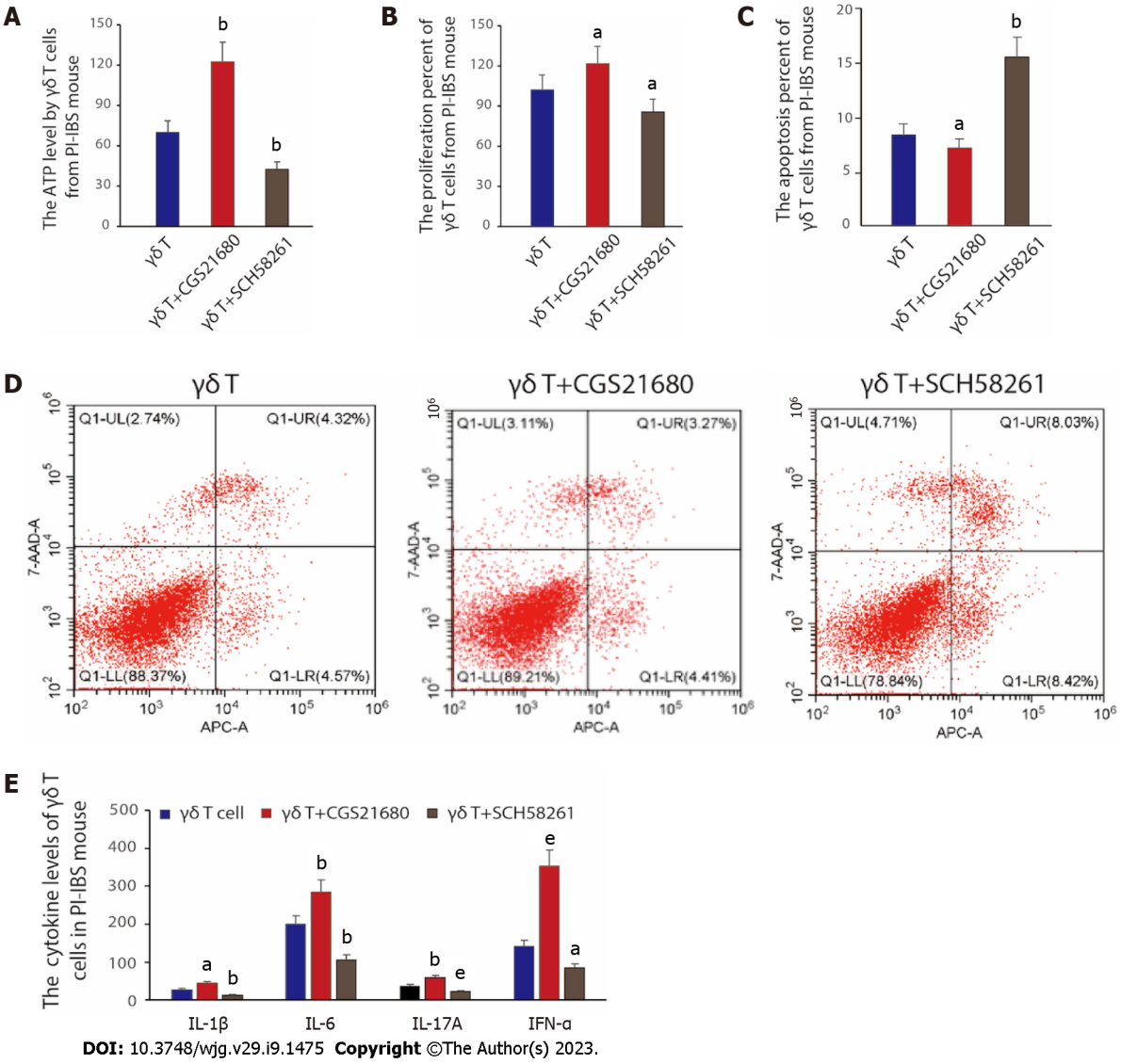
To further clarify the effect of A2AR on the expression of inflammatory factors, we constructed A2AR-



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Figure 3 γδ T cells' isolation and functional evaluation. A-C: Based on the results of fluorescence-activated cell sorting, γδ T cells were effectively extracted and purified. P2, unpurified γδ T cells; P3, purified γδ T cells; D-G: Immunohistochemistry labeling of adenosine 2A receptor (A2AR) expression in γδT cells. Scale bars, 100 μm; H: Western blot analysis of A2AR expression levels in γδ T cells. β-Actin is used as the loading control; I: The quantitive result of A2AR expression level in γδ T cells was analyzed from data shown in H. Results from three times of independently repeated experiments were analysed; J: Reverse transcription polymerase chain reaction analysis of the expression level of A2AR mRNA in γδ T cells. The results were independently repeated three times. A2AR: Adenosine 2A receptor. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the γδ T cell group.

shRNA and inhibited the expression of A2AR by plasmid transfection. The results illustrated that A2AR expression was greatly boosted in the PI-IBS group compared with the control group; when transfected with shRNA, A2AR expression was significantly reduced in the IBS + shRNA group. Moreover, when IBS tissue cells were co-incubated with γδ T cells, the A2AR expression level was further increased. Meanwhile, silencing A2AR downregulated A2AR expression in the IBS + shRNA + γδ T cells group compared with the IBS + γδ T cells group (Figure 6A). After clarifying the silencing efficiency, we examined the effect of silencing A2AR on the expression level of inflammatory factors. From the results in Figure 6B, we can clearly see that the inflammatory factor levels, which were originally higher in the PI-IBS model group, were dramatically reduced due to A2AR knockdown. At the same time, given that the reduced expression of TJ proteins leads to increased intestinal permeability, we also evaluated the altered expression levels of TJ proteins and found that the originally suppressed TJ protein expression in PI-IBS mice was significantly reversed after A2AR silencing (Figure 6C). Taken together, the above findings demonstrate that downregulation of A2AR can reduce γδ T cell-induced inflammatory cytokine production and release.



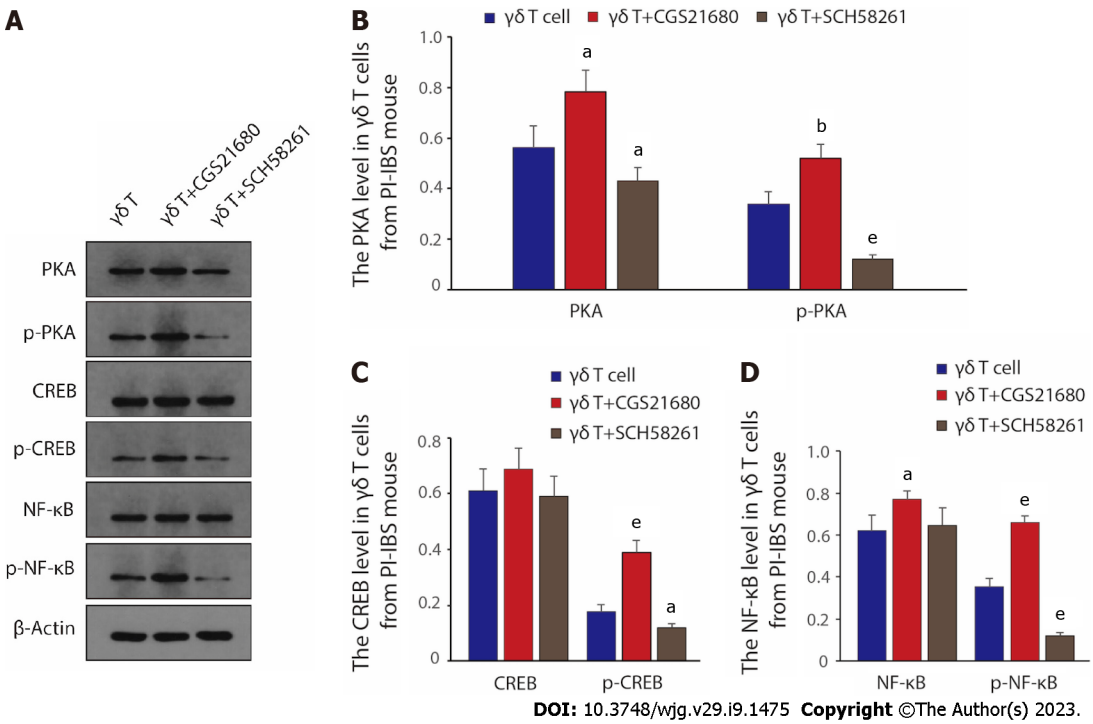
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Figure 4 Functional evaluation of γδ T cells *in vitro*. A: The ATP level changed by differently treated γδ T cells from post-infectious irritable bowel syndrome (PI-IBS) mice. The data was obtained from six mice in each group; B: The proliferation percent of γδ T cells from PI-IBS mouse was analysed through CCK8 assay. The samples were taken from six mice in each group; C: The apoptosis percent of γδ T cells from PI-IBS mouse measured by fluorescence-activated cell sorting. The γδ T cells were taken from six mice in each group; D: Apoptosis rates were detected according to Annexin V and PI double-staining method; E: The cytokines level of IL-1β, IL-6, IL-17A and IFN-α from PI-IBS mice after reinfusing control γδ T cells and γδ T cells treated with CGS21680 or SCH58261, respectively. The data was obtained from six mice in each group. PI-IBS: Post-infectious irritable bowel syndrome. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the γδ T cell group.

DISCUSSION

Persistent intestinal low-degree inflammation occurs in IBS due to complex disorders in the immune system, especially in PI-IBS, leading to minor biochemical and pathological changes but continuous clinical symptoms in patients[34]. Nevertheless, the precise immune regulatory mechanism in PI-IBS remains unknown. We have previously reported that γδ T cells could alleviate PI-IBS through promoting Th17 polarization *via* HSP70 receptor[12]. This study is a follow-up study on the pathogenesis of PI-IBS with the aim of finding new immunomodulatory proteins that directly regulate the intestinal microenvironment and interfere with PI-IBS disease progression.

Few literatures have been reported the relationship between adenosine receptor and PI-IBS. According to our research, an increase in intestinal ATP level is followed by the upregulation of intestinal A2AR in PI-IBS, suggesting that ATP and A2AR may be involved in the pathophysiology of PI-IBS. It is unclear if the quantitative alterations of intestinal A2AR originated from PI-IBS could lead to exacerbated PI-IBS. After being administered with the A2AR antagonist, the digestive tissue of the animals remained unchanged, but their clinical symptoms improved. This discovery contradicted earlier notions that A2AR antagonist exhibits a protective function in inflammation[35]. Moreover, an A2AR antagonist may activate an unidentified pathway to control the equilibrium, and an extended persistence of elevated adenosine levels can be deleterious, contributing to the formation of an immunosuppressed niche that is conducive to the initiation and development of neoplasia[36]. These



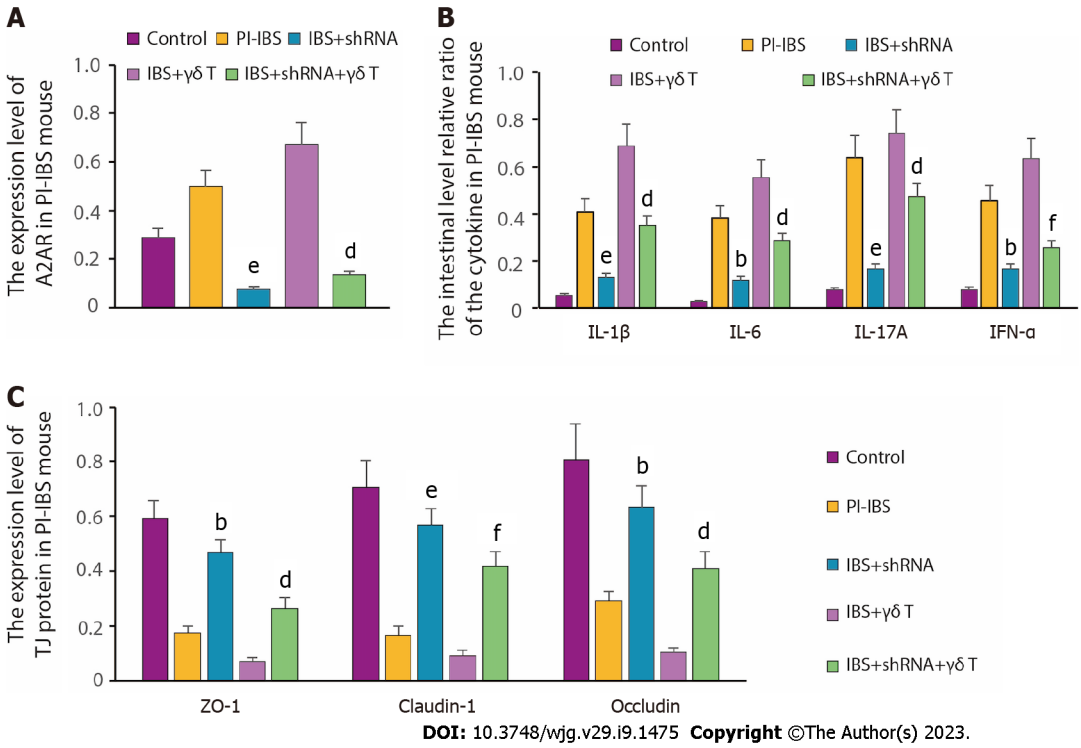
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Figure 5 Adenosine 2A receptor mediated signaling pathway that could regulate the function of γδ T cells. A: The PKA/p-PKA, CREB/p-CREB, NF-κB/p-NF-κB protein levels in γδ T cells of post-infectious irritable bowel syndrome (PI-IBS) mice. β-Actin is used as the loading control; B: The relative PKA level in γδ T cells from mice with PI-IBS; C: The relative CREB level in γδ T cells from PI-IBS mice; D: The relative NF-κB level in γδ T cells from mice with PI-IBS. The results from three times of independently repeated experiments were gathered and analyzed. PI-IBS: Post-infectious irritable bowel syndrome. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the γδ T cell group.

findings also indicate the complexity of PI-IBS.

IL-6, IL-17A, and IFN-α are important pro-inflammatory factors. IL-17A can increase the permeability of cells by binding to specific receptors and can stimulate various types of cells to produce chemokines, IFN-α, and IL-1β to stimulate inflammatory reactions. IFN-α can interrupt the function of intestinal epithelial cells, leading to intestinal epithelial barrier dysfunction[37]. IL-6 is mainly manifested in maintaining regulatory T cells and effector T cells and inhibiting the apoptosis of CD4+ T cells in the inflammatory response[38]. Our study suggested that the levels of pro-inflammatory factors IL-6, IL-17A, and IFN-α were significantly elevated in PI-IBS mice. This indicated that there was a low-grade inflammatory response in the intestinal mucosa, resulting in a defect in the epithelial barrier. The epithelial barrier dysfunction can lead to an increase in intestinal permeability, which further promotes the increase and activation of immune cells[39]. TJ proteins such as ZO-1, Claudin-1, and Occludin form a tight junction structure between adjacent intestinal epithelial cells as the structural basis of the mechanical intestinal barrier, thereby defending against external damaging factors and maintaining intestinal mucosal homeostasis[40,41]. Under the stimulation of severe infection and surgical blows, the tight junction structure between the intestinal epithelium can be destructed, The damaged mechanical barrier and the translocation of intestinal flora into the blood then cause bacteremia and sepsis to stimulate the release of systemic inflammatory factors, leading to systemic inflammatory response syndrome and multiple organ dysfunction[42]. In this study, the expression of ZO-1, Claudin-1, and Occludin was reduced substantially, suggesting that PI-IBS mice had impaired intestinal epithelial barrier function, and were susceptible to stimulation by various etiologies to activate the intestinal epithelial immune system and lead to intestinal inflammatory responses.

The uneven distribution of γδ T cells in normal and inflammatory tissues plays an important role in autoimmunity[43]. In some patients with autoimmune diseases, the proportion of γδ T cells in infiltrating T cells is abnormally increased. γδ T cells are the main source of proinflammatory cytokines IL-17, IL-23, IFN-α, and TGF-β. These inflammatory molecules are responsible for creating an inflammatory environment that enhances disease progression through different pathways[44]. The relationship between different disease phenotypes and γδT cells can be clearly discerned. γδT cells acquire the ability to produce IL-17 during embryonic thymus development, and T cells expressing γδT cell receptors are an important innate source of the pro-inflammatory cytokine IL-17. The potent inflammatory effects of IL-17 are mainly related to its ability to recruit immune cells and synergistic effects with other pro-inflammatory cytokines[45]. Hence, it is no exaggeration to say that γδ T cells play a crucial role in regulating inflammation and the immune response. In our results, we firstly found that the number of γδ T cells markedly increased, accompanied by the increased A2AR expressed on their



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Figure 6 The inflammatory response alteration after adenosine 2A receptor knockdown. A: The altered level of adenosine 2A receptor (A2AR) expression after A2AR-shRNA transfection or γδ T cell reinfusion in post-infectious irritable bowel syndrome (PI-IBS) mice was assessed. The results were obtained from three times of independently repeated experiments; B: The relative ratio of the intestinal cytokine level in PI-IBS mice (n = 6, per group); C: The degree of TJ (tight junction protein) protein expression in PI-IBS mice (n = 6, per group). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the PI-IBS group; $^{c}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* the IBS + γδ T group.

surfaces. Secondly, the agonist of A2AR boosted the quantity of γδ T cells together with upregulated A2AR expression. Thirdly, the A2AR antagonist reduced γδ T cells' number and impaired their function. These findings demonstrated that γδ T cells participated in intestinal inflammation mediated by A2AR.

At present, the anti-inflammatory mechanism of A2AR mainly includes: (1) Inhibition of neutrophils and release of peroxidase[46,47]; (2) promotion of the production of IL-10 by monocytes and macrophages; (3) inhibition of the release of IL-12 and TNF-α[48]; and (4) phosphorylation of cAMP response element-binding (CREB) protein by activating the cAMP-PKA pathway, in which phosphorylated CREB competes with the p65 subunit of NF-κB and the NF-κB coactivator CREB-binding protein combined to inhibit the transcriptional regulation of NF-κB on target genes, thereby blocking the production of inflammatory mediators[49,50]. Excessive release of inflammatory mediators is the main mechanism that causes uncontrolled inflammatory responses in the body. Therefore, the A2AR-cAMP-PKA-CREB-NF-κB signaling pathway is the main mechanism and classical pathway for A2AR mediated function of γδ T cells to inhibit inflammation. Furthermore, we revealed that the A2AR agonist CGS21680 could increase both protein and mRNA expression levels of PKA, CREB and NF-κB, and their phosphorylation activation levels were elevated as well in γδ T cells from PI-IBS mice. Apart from that, we also observed the opposite effects after the treatment of A2AR antagonist SCH58261 in γδ T cells compared with results in A2AR agonist CGS21680 treatment group. Thus, we illustrated that the A2AR-PKA-CREB-NF-κB pathway is the crucial intracellular signaling pathway for A2AR-induced γδ T cells' role in PI-IBS. However, whether there are other signaling pathways at play still needs to be studied.

Furthermore, we decreased the A2AR expression with LV-A2AR-shRNA, which alleviated the symptoms of PI-IBS in model mice. Meanwhile, we then reinfused the γδ T cell to PI-IBS mice, the disease became severer. Interestingly, when the PI-IBS mice reinfused with sh-A2AR γδ T cells, the severity of their clinical symptoms was lessened. Besides, we also observed the alterations in the cytokines and TJ protein expression, and the results were in accordance with the changes in the clinical symptoms. These data suggested that γδ T cells participated in PI-IBS *via* the A2AR mediated signaling pathway.

Our study included a few drawbacks as well. The type of visceral hypersensitivity was not investigated in PI-IBS mice, and the water content from the feces of mice was not examined. Due to the difficulties of isolating T cells directly from the gut, we utilized spleen-isolated T cells, likely resulting in a greater degree of variety than their intestinal counterparts.

CONCLUSION

In the present study, we found higher expression of A2AR in PI-IBS mice than normal mice, which initially suggested the relevance of A2AR to PI-IBS disease development. Further mechanistic studies demonstrated that A2AR, which located on the surface of γδ T cells, could regulate the function of γδ T cells. Notably, A2AR regulated T cell viability and increased the secretion of inflammatory factors, mainly through the PKA/CREB/NF-κB signaling pathway, which followed by the progression of PI-IBS. Fortunately, the application of A2AR antagonists could markedly reduce the inflammatory response and improve PI-IBS symptoms by regulating the function of γδ T cells.

In summary, through our study, we identified A2AR, a key protein that promotes disease progression in PI-IBS, and demonstrated the feasibility of antagonizing A2AR to intervene in PI-IBS, thus providing a new therapeutic target for PI-IBS treatment.

ARTICLE HIGHLIGHTS

Research background

Persistent low level of inflammation due to immune dysfunction is regarded as one of the prime pathogenic mechanisms of post-infectious irritable bowel syndrome (PI-IBS). γδ T cells play a key role in innate and adaptive immunity. Adenosine and its receptors expressed on γδ T cells are involved in intestinal inflammation and immune regulation.

Research motivation

To unveil the role of γδ T cells regulated by adenosine 2A receptor (A2AR) in the pathogenesis of PI-IBS.

Research objectives

This study aims to investigate the role of A2AR in γδ T cells and γδ T cells in PI-IBS.

Research methods

A PI-IBS mouse model was established with Trichinella spiralis (*T. spiralis*) infection. Intestinal A2AR and A2AR in γδ T cells were detected through immunohistochemistry, and inflammatory cytokines were detected through western blot. The role of A2AR on isolated γδ T cells, including γδ T cell proliferation, apoptosis and γδ T cell-mediated cytokine secretion, was assessed *in vitro*. A2AR expression in γδ T cells was determined by western blot and reverse transcription polymerase chain reaction (RT-PCR). Mice were injected with A2AR agonist or A2AR antagonist and cultured γδ T cells were also reinfused into the animals, and then the above parameters and clinical features were examined again. In addition, alterations in A2AR-related signaling pathway molecules were detected by western blot and RT-PCR.

Research results

The expression levels of ATP and A2AR were increased in PI-IBS mice ($P < 0.01$), and inhibition of A2AR further enhanced the clinical features of PI-IBS, as reflected by the abdominal withdrawal reflex and colonic transport test results. The development of PI-IBS was associated with an increase in intestinal γδ T cells and cytokines including interleukin-1 (IL-1), IL-6, IL-17A and interferon-α (IFN-α). In addition, γδ T cells obtained by purification *in vitro* could express A2AR and promote IL-1, IL-6, IL-17A and IFN-α secretion, which is also regulated by A2AR agonists and antagonists. We also found that A2AR antagonists improved γδ T cell function through the PKA/CREB/NF-κB signaling pathway.

Research conclusions

Our results suggested that A2AR contributes to the development of PI-IBS after *T. spiralis* infection *via* the PKA/CREB/NF-κB signaling pathway by γδ T cells.

Research perspectives

Hypo-inflammation caused by immune dysfunction is considered to be one of the main pathogenic mechanisms of PI-IBS. In this study, we discovered that A2AR on the surface of γδ T cells can regulate the function of γδ T cell, thereby increasing inflammatory factor secretion and promoting PI-IBS progression. Luckily, the utilization of A2AR antagonists can improve PI-IBS symptoms by promoting γδ T cells' function. Through our study, we identified A2AR, a key protein that promotes PI-IBS disease progression, and demonstrated the feasibility of antagonizing A2AR to intervene in PI-IBS, thus providing a novel therapeutic target and an effective intervention strategy for PI-IBS treatment.

FOOTNOTES

Author contributions: Dong LW, Chen YY, and Chen CC contributed equally to this manuscript and should be as the co-first authors. Lan C, Sun DM, Dong LW, Chen YY, and Chen CC contributed to the conception and design; Lan C, Dong LW, Chen YY, and Chen CC contributed to development of methodology; Dong LW, Chen YY, Chen CC, Ma ZC, Fu J, Huang BL, and Liu FJ contributed to acquisition of data (provided animals, acquired and managed patients, provided facilities, *etc.*), analysis and interpretation of data (*e.g.*, statistical analysis, biostatistics, computational analysis); Dong LW, Liang DC, and Sun DM contributed to writing, review, and/or revision of the manuscript; Chen YY, Chen CC, and Liang DC contributed to the administrative, technical, or material support (*i.e.*, reporting or organizing data, constructing databases); Lan C supervised the study; all authors have read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Supply and quality of colonoscopy according to the characteristics of gastroenterologists in the French population-based colorectal-cancer screening program

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Abstract

BACKGROUND

Since its complete roll-out in 2009, the French colorectal cancer screening program (CRCSP) experienced 3 major constraints [use of a less efficient Guaiac-test (gFOBT), stopping the supply of Fecal-Immunochemical-Test kits (FIT), and suspension of the program due to the coronavirus disease 2019 (COVID-19)] affecting its effectiveness.

AIM
To describe the impact of the constraints in terms of changes in the quality of screening-colonoscopy (Quali-Colo).

METHODS
This retrospective cohort study included screening-colonoscopies performed by gastroenterologists between Jan-2010 and Dec-2020 in people aged 50-74 living in Ile-de-France (France). The changes in Quali-colo (Proportion of colonoscopies performed beyond 7 mo (Colo_7 mo), Frequency of serious adverse events (SAE) and Colonoscopy detection rate) were described in a cohort of Gastroenterologists who performed at least one colonoscopy over each of the four periods defined according to the chronology of the constraints [gFOBT: Normal progress of the CRCSP using gFOBT (2010-2014); FIT: Normal progress of the CRCSP using FIT (2015-2018); STOP-FIT: Year (2019) during which the CRCSP experienced the cessation of the supply of test kits; COVID: Program suspension due to the COVID-19 health crisis (2020)]. The link between each dependent variable (Colo_7 mo; SAE occurrence, neoplasm detection rate) and the predictive factors was analyzed in a two-level multivariate hierarchical model.

RESULTS
The 533 gastroenterologists (cohort) achieved 21509 screening colonoscopies over gFOBT period, 38352 over FIT, 7342 over STOP-FIT and 7995 over COVID period. The frequency of SAE did not change between periods (gFOBT: 0.3%; FIT: 0.3%; STOP-FIT: 0.3%; and COVID: 0.2%; $P = 0.10$). The risk of Colo_7 mo doubled between FIT [adjusted odds ratio (aOR): 1.2 (1.1; 1.2)] and STOP-FIT [aOR: 2.4 (2.1; 2.6)]; then, decreased by 40% between STOP-FIT and COVID [aOR: 2.0 (1.8; 2.2)]. Regardless of the period, this Colo_7 mo's risk was twice as high for screening colonoscopy performed in a public hospital [aOR: 2.1 (1.3; 3.6)] compared to screening-colonoscopy performed in a private clinic. The neoplasm detection, which increased by 60% between gFOBT and FIT [aOR: 1.6 (1.5; 1.7)], decreased by 40% between FIT and COVID [aOR: 1.1 (1.0; 1.3)].

CONCLUSION
The constraints likely affected the time-to-colonoscopy as well as the colonoscopy detection rate without impacting the SAE's occurrence, highlighting the need for a respectable reference time-to-colonoscopy in CRCSP.

Key Words: Colorectal cancer screening; Screening colonoscopy; Faecal immunochemical test; Guaiac faecal occult blood test; Quality of colonoscopy; Severity of tumor lesions

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Core Tip: The study showed that the detection rate of colonoscopy dropped significantly in France during the years 2019 and 2020, probably due to the coronavirus disease health crisis. The risk of a long delay (> 7 mo) in performing the colonoscopy was twice as high in a public hospital compared to colonoscopies performed in a private endoscopy practice. The constraints likely affected the time to colonoscopy as well as the colonoscopy detection rate without impacting the occurrence of serious adverse events.

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INTRODUCTION

The impact of the Screening program on controlling colorectal cancer (CRC) morbidity and mortality has been widely proved[1-4]. But since its complete roll-out in France in 2009, the population-based colorectal cancer screening program (CRCSP) has continued to face constraints affecting its effectiveness. Despite the existence of the fecal immunochemical test (FIT) in certain European programs (*i.e.*, Italy, Czech Republic) when the program roll-out was completed in France[5], the health authority chose the Guaiac Hemoccult II test® (gFOBT). It later turned out that gFOBT only identified 50% of colorectal cancer (CRC) lesions and a third of adenomas[6], which led some GPs to be wary of it, at the

risk of seeing some of their patients fall through the cracks[6,7].

To consider this first constraint induced using a low sensitivity/specificity screening test, the health authority decided to replace gFOBT in 2015, with the FIT (Threshold set at 150 ng hemoglobin/mL of stool, "Institut National du Cancer", www.e-cancer.fr). While admitting an improvement in participation with FIT compared to gFOBT, most studies published in France have confirmed the high sensitivity (detection of advanced adenomas and CRC) of FIT and its better acceptability by the population and GPs[8-12]. This performance of the FIT inevitably leads to an increase in colonoscopy requests in the screened population and subsequently to an extension of the time to colonoscopy after a positive FIT result[13]. However, these analyses of the time to colonoscopy only considered the characteristics of the target population without any adjustment to the characteristics of the colonoscopy supply.

On April 25, 2018, the Paris Administrative Court cancelled, during an appeal session, the contract concluded in 2014 between the Health Insurance Agency and the Cerba-Daklapack® consortium (www.slbc.fr). This contract, which related to the supply of screening test kits and the laboratory analysis of the tests carried out, had thus been cancelled only three years after the introduction of the FIT in CRCSP. This legal and administrative confusion led to a market shutdown between March and September 2019. In the Ile-de-France (IDF) region, this shutdown led to a drastic decrease in the number of tests carried out in 2019, compared to forecasts (annual activity report 2019).

Only a few months after the resumption of the test kits' market, the World Health Organization (WHO) announced the pandemic of COVID-19[14]. This pandemic constraint required a relocation of health care resources to control this global health crisis. Screening programs, in particular the CRCSP, were suspended in many countries. The aim of this study was to describe the impact of the constraints listed above in terms of changes to the quality of screening colonoscopies (Quali-colo) in a cohort of gastroenterologists (GEs) practicing in IDF.

MATERIALS AND METHODS

This retrospective cohort study included all screening colonoscopies, performed between 01/01/2010 and 31/12/2020 by GEs in the IDF region and collected by the eight sites (Paris, Seine-et-Marne, Yvelines, Essonne, Hauts-de-Seine, Seine-Saint-Denis, Val-de-Marne and Val-d'Oise) of the IDF CRCSP Coordination Centre (CRCDC-IDF). These screening colonoscopies were performed following a positive screening test in people aged 50-74, living in IDF, France.

Considering the chronology of the constraints in the CRCSP, four periods for carrying out the colonoscopy were distinguished (Figure 1). The first period (gFOBT) corresponded to the five years (2010-2014) of normal progress of the CRCSP using gFOBT. The second period (FIT) corresponded to the four years (2015-2018) of normal progress of the CRCSP using FIT. The third (FIT-STOP) corresponded to the year (2019) during which the CRCSP experienced the cessation of the supply of test kits and the fourth (COVID) corresponded to the program suspension due to the COVID-19 health crisis (2020).

The supply of screening colonoscopy was described by the number and type of practice of GEs practicing in IDF and having performed a screening colonoscopy in a person living in IDF. The Quali-colo was described in terms of time to colonoscopy, yield of colonoscopy and frequency of undesirable events (incidents/accidents, incomplete colonoscopy, refusal of 2nd colonoscopy).

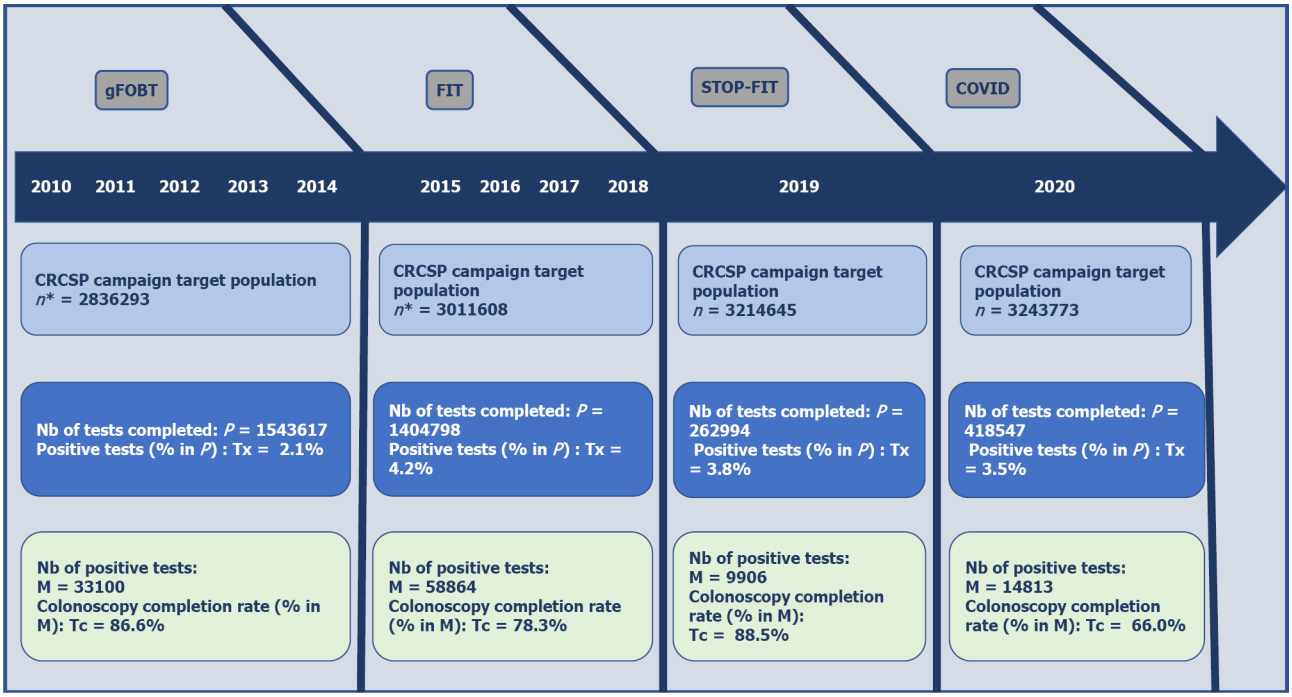
Descriptive and evolutive analyses (supply and Quali-colo) were carried out between the periods (gFOBT, FIT, FIT-STOP, and COVID). These changes were first described according to the characteristics of the GEs who performed the screening colonoscopies. Secondly, the impact of constraints was described in terms of changes in Quali-colo indicators between the four periods, in a cohort of GEs (Cohort-GE) who performed at least one colonoscopy in each of the four periods.

Screening organization and study data collection

The National Council of the Order of Physicians (Research and Statistics Study Department) provided the medical demographic data. Screening data were extracted from CRCDC-IDF departmental databases. Over the study period, the CRCSP campaigns were organized following the CRCSP specifications[15,16]. As a preliminary to each campaign in each study department, an update of the files of eligible people was made after the transmission of individual data by the partners (Health Insurance plans, Medical Information Services of hospitals, Pathologists, GEs, Surgeons, GPs, patients). Anyone who had a screening test did not need a screening colonoscopy if the test result was negative. In case of a positive test result, the person was subsequently invited five years after a normal colonoscopy or excluded from the CRCSP after a positive colonoscopy result (polyp or CRC).

Definition of variables

The screening colonoscopy (complete or incomplete) was considered completed only if the result was provided with or without a completion date. When the completion date was provided, the time to screening colonoscopy was expressed as the number of months between the date of completion of the screening test and the date of completion of the colonoscopy. In the cases where several colonoscopies



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Figure 1 Evolution of the colorectal cancer screening program indicators (target population of the campaigns, number of tests carried out, number and proportion of positive tests, rate of completion of colonoscopy) over the 4 study periods (guaiac fecal occult blood test, fecal immunochemical test, STOP-fecal immunochemical test, and COVID). The asterisk (*) is the target population at the start of the period. The colonoscopy completion rate was estimated based on data extraction as of January 31, 2022. gFOBT: Guaiac fecal occult blood test; COVID: Coronavirus disease; FIT: Fecal immunochemical test; Nb: Number; CRCSP: Colorectal cancer screening program.

were carried out to investigate the same positive test, the time to screening colonoscopy was that related to the first colonoscopy. The proportion of screening colonoscopies with an abnormally long time to access colonoscopy (Long-delay-colo) was estimated by the frequency of colonoscopies performed beyond a 7-mo delay among the screening colonoscopies for which the completion date was provided. This delay threshold considers the fact that the risk of colorectal cancer is increased by about 40% for any colonoscopy performed after a waiting period of 7-12 mo[17].

The screening colonoscopy was complete when the colon was examined until crossing the Bauhin valve. The reasons for an incomplete colonoscopy were: Insufficient preparation, Anatomical (dolichocolon, Presence of an obstructive lesion requiring a second colonoscopy or surgery). The accidents related to screening colonoscopy were: exterior hemorrhage with or without transfusion, perforation, death. Incidents related to anesthesia or general condition (cardiorespiratory disorders) were distinguished from those related to endoscopy (*i.e.*, difficulty crossing a cul-de-sac, placement of clips to stop bleeding after a polypectomy). The proportion of serious adverse events (SAEs) was estimated by the frequency of screening colonoscopies during which an incident/accident was notified.

The screening colonoscopy was classified as positive when a neoplasm (Polyp/adenoma/CRC) was discovered, negative if not. The screening colonoscopy detection rate (yield of colonoscopy) was estimated by the proportion of positive colonoscopies among the screening colonoscopies performed. The CRC and polyps/adenomas diagnoses were those coded C18-C20 and D12 according to the 10th version of the WHO International Classification of Diseases (ICD10)[18]. The CRC was considered "seen at colonoscopy" when an ulcerative-budding/ulcerative-necrotizing lesion was described by the GE. The high-risk polyps were adenomatous or scalloped polyps with a diameter of ≥ 10 mm (except hyperplastic polyps), high-grade dysplasia adenomas, villous or tubulo-villous adenomas. The TNM classification[19] has been used to define CRC severity. Any CRC ≥ T3 (subserous invaded) or ≥ N1 (at least one regional node invaded) or M1 (with metastasis) was considered severe CRC.

For each GE practicing in IDF region, having performed at least one screening colonoscopy, the factors studied were: (1) The existence of a gastroenterology consultation carried out before the screening colonoscopy completion date; (2) the annual number of screening colonoscopies performed (1, 2-30, 31-100, and > 100 colonoscopies); (3) the place of performance of the screening colonoscopy (1-Private clinic in the IDF; 2-Private hospitals in the IDF; 3-Public hospital in the IDF including: The Public Assistance of Paris hospitals -APHP-, Other public hospitals in the IDF including army hospitals and municipal health centers). The colonoscopies performed by GEs practicing in ≥ 2 locations, the locations of which had not been specified (n = 2), were attributed to the locations most frequented by these GEs over the period. Similarly, Colo for which the location was specified but for which the GEs were not

specified (n = 6), were attributed to the GEs who performed the greatest number of colonoscopies on the location and over the period. Colo performed in a country other than France were classified as "Place Unspecified". Colonoscopies performed in another region of France were classified "Outside-IDF"; (4) the annual number of colonoscopy locations (1 Location, ≥ 2 Locations); (5) the density of GEs in the municipality where the GE performed the screening colonoscopy. The density (D) of GEs was estimated as number of GEs/100000 inhabitants. Each colonoscopy year, with reference to a regional average density (M) and standard deviation (SD). Low density of GE was: D < M-SD, average-density of GE was: D in M ± SD, high density of GE was: D > M + SD; (6) the seniority of the GE (for any year "A", the GE having no screening colonoscopy in the years prior to "A" was considered a new GE); (7) the residence of the CRCSP target patient treated by the GE (1-the Colonoscopy's supply municipality, 2-other municipality in the Colonoscopy's supply department, 3-other IDF departments). As a reminder, in 2018, The National Institute of Statistics and Economic Studies (INSEE) counted 1267 municipalities in IDF in addition to the city of Paris; and (8) the age of the CRCSP target patient treated by the GE (50-54, 55-59, 60-64, 65-69, and ≥ 70 years).

Statistical analysis

The proportions (Colo performed within one month or after a waiting delay > 7 mo, incomplete and redone Colo, incidents/accidents, positive Colo, high_risk_polyp, CRC seen at Colo, CRC with provided status, severe CRC) were described and compared between periods (gFOBT, FIT, FIT-STOP, and COVID) by the Pearson' Chi-2 test. In the strata defined according to the characteristics of the cohort-GE, the time to perform the screening colonoscopy (in months) was analyzed in terms of average and confidence interval (CI) then, an analysis of variance (ANOVA on repeated measures) was used to compare the average delays between periods (gFOBT, FIT, FIT-STOP *vs* COVID). In the strata defined according to the characteristics of the cohort-GE, the proportions (colonoscopies performed after > 7 mo delay, proportion of SAEs, yield of screening colonoscopy) were compared between periods (gFOBT, FIT, FIT-STOP *vs* COVID) by Cochran's Q test.

The link between each dependent variable (binary variables 0/1: Long-delay-colo; SAEs, Yield of screening colonoscopy) and the predictive factors (annual number of screening colonoscopies performed, Place of performance of the screening colonoscopy, Annual number of colonoscopy locations, Density of GE, Residence of the patient, Age of the patient) was analyzed in a multivariate and two level (colonoscopy and GE) hierarchical regression model. The generalized linear model (family: Bernoulli, link: Logit) with mixed effect was preferred. This multivariate analysis was performed using a model with all covariates regardless of their relationship in univariate analysis. In addition, a strong correlation existed between several covariates (*i.e.*, annual number of screening colonoscopies and Place of performance, Annual number of screening colonoscopies and Municipal density of GEs, Annual number of screening colonoscopies and Period), the model was extended to these terms of interaction between covariates. Only the significant interaction terms (P < 0.05 in univariate analysis) were kept in the final model evaluated by the likelihood ratio test. A biomedical statistician performed the statistical review. All the analyses were carried out at the 5% threshold with version 13 of the STATA software (College Station, TX, United States).

Regulatory issues

Before analysis, all data were anonymized. The screening database had a favourable opinion from the institution that oversees the ethics of data collection ("Commission *nationale de l'informatique et des libertés*": CNIL)[20]. According to the current French legislation, a study that does not change the care of patients did not require the opinion of the Clinical Research Centre's Ethics Committee.

RESULTS

Descriptive and evolutive analyses

Out of a total of 1267 municipalities listed in the IDF region, only 155 municipalities had at least one GE in 2010. This number of municipalities having at least one GE falling from 155 in 2010 to 142 in 2020. In the municipalities having at least one GE, the average annual density of GEs fluctuated between a minimum of 6.3 (in 2014) and a maximum of 6.5 GE/100000 inhabitants over the study period (Table 1).

The gap between the number of GEs registered in the medical demographic database and the number of GEs having performed at least one screening colonoscopy, increased from 134 in 2010 (761 registered *vs* 627 having performed ≥ 1 screening colonoscopy), to 206 in 2015 (776 *vs* 570) before being reduced to 123 in 2019 (798 *vs* 675). The proportion of GEs performing screening colonoscopies at two or more locations varied from 20.6% in 2010 to 13.9% in 2015, then 21.8% in 2019. The proportion of new GEs decreased from 12.6% in 2011 to 7.7% in 2015, then increased to 13.5% in 2016 and further decreased to 4.7% in 2019. In 2016, a total of 727 GEs performed at least one colonoscopy. Among them, 97 GE performed only one screening colonoscopy and 8 GEs exceeded an annual number of 100 screening colonoscopies (Table 1).

Table 1 Evolution of the regional offer in number of gastroenterologists and the number of gastroenterologists having performed at least one colonoscopy, by year of performance of the screening colonoscopy

Year of colonoscopy	Nb of GE in IDF[1]		Number of gastroenterologists who performed a screening colonoscopy[2]												
			Number of GE by seniority		Number of GE by density of GE in the municipality of practice of the GE			Number of GE by place of performance of the colonoscopy			Number of GE by annual number (A) of colonoscopies performed				Total (*n*) of GE in IDF (% GE ≥ 2 location)
	Nb of GE (density)[3]	Nb of municipalities with GE	Senior	New (% in *n*)	Low	Average	High	Private clinic	Private Hop.	Public Hop.	A = 1	A = 2-30	A = 30-100	A > 100	
2010	761 (6.5)	155	627	-	134	85	493	415	114	214	119	473	35	-	627 (20.6)
2011	756 (6.4)	156	534	77 (12.6)	140	71	474	408	117	201	106	465	40	-	611 (17.2)
2012	759 (6.4)	155	538	57 (9.6)	116	79	473	383	112	206	117	454	24	-	595 (16.8)
2013	761 (6.4)	154	539	30 (5.3)	98	92	442	378	115	181	107	448	14	-	569 (16.5)
2014	757 (6.3)	155	522	63 (10.8)	129	75	451	384	106	193	123	448	14	-	585 (17.4)
2015	776 (6.4)	154	526	44 (7.7)	117	53	449	379	103	178	140	419	11	-	570 (13.9)
2016	784 (6.5)	154	629	98 (13.5)	128	65	628	432	143	312	97	447	175	8	727 (18.8)
2017	793 (6.5)	152	642	72 (10.1)	142	56	603	418	142	312	93	486	134	1	714 (19.9)
2018	799 (6.5)	149	665	64 (8.8)	141	51	626	424	151	312	100	488	139	2	729 (20.7)
2019	798 (6.5)	147	643	32 (4.7)	123	63	574	388	152	287	92	512	71	-	675 (21.8)
2020	802 (6.5)	142	619	76 (10.9)	147	50	582	412	162	265	124	475	96	-	695 (19.7)

[1]Number of gastroenterologists (GE) registered in the region (source: National Council of the Order of Physicians).
[2]Number of gastroenterologists who performed a screening colonoscopy during the calendar year (regardless of the type of test and regardless of the date of the screening test).
[3]Density in Number of GE/100000 inhabitants: Regional average density (5.5 à 7.5 GE/100000 inhabitants) Low density of GE (< 5.5 GE/100000 habitants) and high density of GE (> 7.5 GE/100000 inhabitants).
GE: Gastroenterologist; Hop: Hospital; IDF: Ile-de-France; Nb: Number.

In 2011, out of a total of 6428 colonoscopies performed in IDF, the proportion of colonoscopies performed by new GEs was 2.0%, the proportion of colonoscopies performed in a municipality with a high density of GEs was 62.2%, the proportion of colonoscopies performed in a public hospital was 12.5%. In 2016, 1041 screening colonoscopies were performed by the GEs having an annual volume of > 100 screening colonoscopies and 9148 (58.9%) screening colonoscopies were performed by the GEs having an annual volume of 30-100 screening colonoscopies. Compared to 2010 (1.7%), the proportion of screening colonoscopies performed outside the IDF region was significantly higher in 2020 (2.5%; $P < 0.0001$). Similarly, compared to 2019 (16.8%), the proportion of screening colonoscopies performed in public hospitals decreased significantly in 2020 (13.0%, $P < 0.0001$) (Table 2).

Table 2 Evolution of the number of colonoscopies performed according to the characteristics of the gastroenterologist, by year of performance of the screening colonoscopy, *n* (%)

Year of colonoscopy	Number of colonoscopies performed according to GE characteristics														
	Number of colonoscopies by seniority of GE		Number of colonoscopies by density of GE in the municipality of practice of the GE[1]			Number of colonoscopies by place of performance of the colonoscopy			Number of colonoscopies by GE's annual number (A) of colonoscopies performed				Total		
	Senior	New	Low	Average	High	Clinic	Private Hop.	Public Hop.	A = 1	A = 2-30	A = 31-100	A > 100	Nb (*n*) of Colo performed in IDF (average Nb of Colo by GE)	Nb of Colo with place specified (% outside IDF)	Nb of Colo (% Place unspecified)
2010	6059	-	1535	900 (14.9)	3624 (59.8)	4507	830 (13.7)	722 (11.9)	119	4493 (74.2)	1447 (23.9)	-	6059 (11)	6161 (1.7)	6441 (4.4)
2011	6300	128 (2.0)	1684	712 (11.1)	4032 (62.7)	4677	946 (14.7)	805 (12.5)	106	4578 (71.2)	1744 (27.1)	-	6428 (12)	6543 (1.8)	6928 (5.6)
2012	5355	76 (1.4)	1186	766 (14.1)	3479 (64.1)	3818	830 (15.3)	783 (14.4)	117	4284 (78.9)	1030 (19.0)	-	5431 (11)	5533 (1.8)	5852 (5.5)
2013	4309	47 (1.1)	1045	737 (16.9)	2574 (59.1)	3156	660 (15.2)	540 (12.4)	107	3725 (85.5)	524 (12.0)	-	4356 (9)	4409 (1.2)	4712 (6.4)
2014	4320	132 (3.0)	1104	611 (13.7)	2737 (61.5)	3199	650 (14.6)	603 (13.5)	123	3718(83.5)	611 (13.7)	-	4452 (9)	4515 (1.4)	4746 (4.9)
2015	3712	63 (1.7)	879	446 (11.8)	2450 (64.9)	2692	604 (16.0)	479 (12.7)	140	3198 (84.7)	437 (11.6)	-	3775 (8)	3818 (1.1)	4034 (5.4)
2016	15196	333 (2.1)	3406	1862 (12.0)	10261 (66.1)	10886	2527 (16.3)	2116 (13.6)	97	5243 (33.8)	9148 (58.9)	1041	15529 (25)	15811 (1.8)	16651 (5.0)
2017	11519	192 (1.6)	2876	1262 (10.8)	7573 (64.7)	7919	1937 (16.5)	1855 (15.8)	93	5370 (45.9)	6137(52.4)	111	11711 (18)	11920 (1.8)	12345 (3.4)
2018	12181	164 (1.3)	2758	1190 (9.6)	8397 (68.0)	8233	2300 (18.6)	1812 (14.7)	100	5331 (43.2)	6684 (54.1)	230	12345 (19)	12602 (2.0)	13057 (3.5)
2019	8189	98 (1.2)	1582	932 (11.3)	5773 (69.7)	5365	1532 (18.5)	1390 (16.8)	92	5261 (63.5)	2934 (35.4)	-	8287 (13)	8487 (2.4)	8767 (3.2)
2020	9103	158 (1.7)	2088	755 (8.2)	6418 (69.3)	6654	1900 (20.5)	1199 (13.0)	124	5049 (54.5)	4088 (44.1)	-	9261 (15)	9501 (2.5)	9793 (3.0)

[1]Density in Number of GE/100000 inhabitants: Regional average density (5.5 à 7.5 GE/100000 inhabitants), low density of GE (< 5.5 GE/100000 habitants) et High density of GE (> 7.5 GE/100000 inhabitants).
Colo: Screening colonoscopy; GE: Gastroenterologist; Hop.: Hospital; IDF: Ile-de-France; Nb: Number.

Overall, the time to screening colonoscopy was significantly longer over STOP-FIT (gFOBT: 2.6 ± 2.9 *vs* FIT: 3.0 ± 3.0; STOP-FIT: 3.9 ± 3.9, COVID: 3.5 ± 3.9, $P < 0.0001$). Over the gFOBT period, 3.1% of the 28679 colonoscopies performed were incomplete (20.7% were redone) for reasons: Anatomical (60.6%), insufficient preparation (16.1%). The proportion of incomplete and redone colonoscopies was significantly higher over FIT ($P < 0.001$). Although one case of death was reported during the gFOBT period, the proportion of adverse events was not significantly related to the period (0.05). The proportion of cancers seen at colonoscopy was lower over FIT (gFOBT: 61.4%, *vs* FIT: 55.2% or STOP-FIT: 57.5% or COVID: 56.1%; $P < 0.0001$) (Table 3).

Table 3 Quality indicators and results of colonoscopies by period of performance of colonoscopy in people aged 50-74, residing in Ile-de-France, *n* (%)

Quality indicator	Period				
	gFOBT	FIT	STOP-FIT	COVID	*P* value[1]
Total number (*n*) of colonoscopies	28679	46087	8767	9783	
Existence of a GE consultation before colonoscopy					
Nb (A) colonoscopies with date of consultation	5267 (18.4)	1517 (3.3)	406 (4.6)	198 (2.0)	$< 10^{-3}$
Date of consultation ≠ Date of colonoscopy					
Nb colonoscopies of which date of consultation ≠ colon date (% in A)	298.4 (56.7)	883 (58.2)	402 (99.0)	191 (96.5)	$< 10^{-3}$
Time to colonoscopy					
Average (in mean ± SD)	2.6 ± 2.9	3.0 ± 3.0	3.9 ± 3.9	3.5 ± 2.9	$< 10^{-3*}$
Number of colonoscopies performed within one month	4957 (17.3)	4572 (9.9)	458 (5.2)	726 (7.4)	$< 10^{-3}$
Number of colonoscopies performed beyond 7 mo	1520 (5.3)	2949 (6.4)	1034 (11.8)	933 (9.5)	$< 10^{-3}$
Complete colonoscopy					$< 10^{-3}$
Nb colonoscopies without information on performance	1263 (4.4)	2360 (5.1)	410 (4.7)	432 (4.4)	
Number of complete colonoscopies	26530 (92.5)	41695(90.5)	8004 (91.3)	8981 (91.8)	
Nb (B) of incomplete colonoscopies	886 (3.1)	2032 (4.4)	357 (4.1)	376 (3.8)	
Reasons for incomplete colonoscopies					$< 10^{-3}$
Unspecified: *n* (% in B)	206 (23.3)	617 (30.4)	109 (30.5)	114 (30.3)	
Anatomical reason/Obstruction by lesion: *n* (% in B)	537 (60.6)	845 (41.6)	150 (42.0)	161 (42.8)	
Insufficient preparation: *n* (% in B)	143 (16.1)	570 (28.1)	98 (27.5)	101 (26.9)	
Redone incomplete colonoscopy					
Number of redone colonoscopies (% B)	183 (20.7)	960 (47.2)	158 (44.3)	163 (43.3)	$< 10^{-3}$
Frequency of incidents					0.14
No incidents reported: *n*	28873 (99.6)	45947 (99.7)	8740 (99.7)	9763 (99.8)	
Related to anaesthesia/general condition: *n*	18 (0.06)	24 (0.05)	3 (0.03)	2 (0.02)	
Related to endoscopy: *n*	88 (0.3)	116 (0.3)	22 (0.3)	18 (0.2)	
Frequency of accidents					0.17
No accidents reported: *n*	28589 (99.7)	45970 (99.8)	8749 (99.8)	9763 (99.8)	
Suspected complication: *n*	24 (0.08)	23 (0.05)	5 (0.04)	3 (0.03)	
Exterior bleeding: *n*	57 (0.2)	66 (0.1)	14 (0.2)	12 (0.1)	
Perforation: *n*	8 (0.03)	28 (0.06)	3 (0.03)	4 (0.04)	
Deaths: *n*	1 (0.0)	0	0	0	
Colonoscopies results					
Detection rate: Nb of lesions	14857 (51.8)	29843 (64.8)	5565 (63.5)	5967 (60.1)	$< 10^{-3}$
Nb Polyps (% HRP)	12947 (44.2)	26624 (56.4)	5040 (53.3)	5425 (51.8)	$< 10^{-3}$
Nb of CRC (% CRC seen at colonoscopy)	1910 (61.4)	3219 (55.2)	525 (57.3)	542 (56.1)	$< 10^{-3}$
% CRC with severity stage specified among Nb CRC[2]	90.3	80.5	74.3	72.3	$< 10^{-3}$
Nb CRC with severity stage specified (% severe CRC)[2]	1724 (50.7)	2592 (40.9)	390 (39.5)	392 (39.5)	$< 10^{-3}$

[1]Pearson's χ^2/Fisher's exact test of proportion or Fisher's *F* test (ANOVA).
[2]Any CRC ≥ T3 (subserous invaded) or ≥ N1 (at least one regional node invaded) or M1 (with metastasis) was considered severe colorectal cancer.
CRC: Colorectal cancer; FIT: Fecal immunochemical test; GE: Gastroenterologist; gFOBT: Guaiac fecal occult blood test; HRP: High risk polyps (advanced adenoma); Nb: Number.

Changes in Quali-colo indicators between the four periods, in a cohort of GEs

The cohort of 533 GE achieved 21509 Screening colonoscopies over the gFOBT period, 38352 over FIT, 7342 over STOP-FIT and 7995 over the COVID period. In this cohort, the difference in time (months) to screening colonoscopy between periods was globally significant [gFOBT: 2.6 (2.5; 2.6) *vs* FIT: 3.0 (2.9; 3.0); STOP-FIT: 3.9 (3.8; 4.0) and COVID: 3.5 (3.4; 3.6); $P < 0.0001$]. The average time to colonoscopy was longer in public hospitals compared to clinics or private hospital, regardless of the period. This average time was paradoxically shorter over the COVID period compared to the STOP-FIT period, regardless of the type of establishment [in STOP-FIT clinic: 3.7 (3.6; 3.7) *vs* COVID: 3.4 (3.3-3.5) in public hospitals STOP-FIT: 5.1 (4.7-5.9) *vs* COVID: 4.2 (3.8; 4.7)]. The average time to colonoscopy was significantly lower among GEs practicing in low-density areas of GEs compared to those practicing in high-density areas of GEs, over the gFOBT and FIT periods, conversely, depending on the density area the confidence intervals were not significant over the STOP-FIT and COVID periods (Table 4).

Regardless of the GE's characteristics, the proportion of screening colonoscopy performed in > 7 mo delay was significantly higher over STOP-FIT ($P < 0.001$). The proportion of colonoscopies performed in > 7 mo delay was higher in public hospitals compared to clinics and private hospitals, regardless of the period ($P < 0.001$ in each period). This proportion of colonoscopies performed in > 7 mo delay decreased during the COVID period compared to the STOP-FIT period, regardless of the place of colonoscopy $P < 0.001$ for each place). The proportion of colonoscopies performed in > 7 mo delay was higher in the 50-54 age group, regardless of the period $P < 0.001$ in each period) (Table 5).

Whatever the characteristics of the Cohort-GE, the decline in colonoscopy detection rate was significant between the FIT and COVID period (Table 6). The risk of having a long delay to colonoscopy was twice as high for screening-colonoscopy performed in a public hospital [adjusted odds ratio (aOR): 2.1 (1.3; 3.6)] compared to screening colonoscopy performed in a private IDF clinic. Except for the patient's age, the risk of adverse events was not related to any other predictive factor. Compared to patients aged 50-54, patients aged 70 had a 70% increased risk of neoplasm detection. The risk of neoplasm detection decreased by about 40% between the periods FIT [aOR: 1.6 (1.5; 1.7)] and COVID [aOR: 1.1 (1.0; 1.3)] (Table 7).

DISCUSSION

The European guide for quality assurance of colorectal cancer screening recommends performing a colonoscopy within 31 d following a positive test result[21]. In our Cohort-GE, if the increase in the time to screening colonoscopy between the first and the second period was attributable to the introduction of FIT, its increase after the second period was attributable to the malfunction of the program due to the slowdown of the kit market and the COVID-19 health crisis. There is certainly no relationship between the kit market and the colonoscopy offer, but the unexplained increase in the time to perform colonoscopy during a year that saw a market slowdown can be explained factually by this market crisis. The hypothesis would be that general practitioners reacted to the market crisis by relaxing the program, in particular the follow-up of people who had a positive test. Indeed, in France, in addition to the distribution of the test kit, the training doctors are real facilitators of access to colonoscopy (helping the patient to make an appointment with a gastroenterologist, motivating the patient to have the colonoscopy). This hypothesis is confirmed by the slight decrease in the time to colonoscopy in 2020 compared to 2019, despite the COVID-19 health crisis. The year 2020 was moreover affected by this kit market crisis than by the COVID-19 health crisis. Indeed, after the resumption of the kit market in September 2019, several people who had a positive test during the last quarter of 2019 were inevitably the first to be affected by colonoscopy postponements at the start of the first confinement in March 2020. However, the improvement in the time to colonoscopy during the pandemic (compared to the STOP-FIT period) could also be linked to the fact that people have refocused their concerns on their health. Regardless of the characteristics of the Cohort-GE, the screening colonoscopy detection rate dropped significantly between the STOP-FIT and COVID periods, while the proportions of SAEs stayed unchanged.

The long delay to access colonoscopy observed on the gFOBT and FIT periods converges with the results of another French study[22], although it is clearly higher than those observed elsewhere[23,24]. The definition of a reference delay and the obligation of compliance with it by all GEs taking part in CRCSP would effectively reduce the delay in France. This reframing is necessary, especially since the number of GEs is large, but with an increased disparity in terms of the number of screening colonoscopies performed by GEs.

Despite this longer waiting time to colonoscopy, the proportion of colonoscopies during which a SAE was reported did not change between periods. Although high, the frequency of perforations remains lower than that (1.1%) found in Alsace[25]. In the program, there was no nationally standardized forms for collecting screening colonoscopy data. Information concerning the date of consultation before the colonoscopy, or the progress of the examination can sometimes be missed or be considered irrelevant during this collection. Therefore, the low frequency of SAEs reported in this study could be the consequence of under-reporting.

Table 4 Average time (in months) to colonoscopy according to the characteristics of the cohort of gastroenterologists who performed at least one colonoscopy over each of the four periods (guaiac fecal occult blood test, fecal immunochemical test, STOP-fecal immunochemical test, and COVID)

Characteristics of the cohort of gastroenterologists	Nb of GE	Average time (in months) to colonoscopy, by period								P^1
		gFOBT		FIT		STOP-FIT		COVID		
		Nb of Colo	Average, 95%CI	Nb of Colo	Average, 95%CI	Nb of Colo	Average, 95%CI	Nb of Colo	Average, 95%CI	
Overall	533	21509	2.6 [2.5; 2.6]	38352	3.0 [2.9; 3.0]	7342	3.9 [3.8; 4.0]	7995	3.5 [3.4; 3.6]	$< 10^{-3}$
Annual Nb of Colo										
1	201[2]	304	3.1 [2.8; 3.5]	150	3.3 [2.7; 3.8]	38	4.3 [3.1; 4.8]	51	4.0 [3.5; 4.6]	0.08
2-30	481[2]	16819	2.6 [2.5; 2.6]	15970	3.0 [3.0; 3.1]	4887	3.9 [3.8; 4.0]	4211	3.5 [3.4; 3.6]	$< 10^{-3}$
31-100	44[2]	4386	2.4 [2.3; 2.5]	21137	3.0 [2.9; 3.0]	2817	3.8 [3.6; 3.9]	3733	3.5 [3.4; 3.6]	$< 10^{-3}$
> 100	0	0		1095	2.5 [2.3; 2.6]	0		0		
Place of S-colo performance										
Clinic	355[2]	15745	2.4 [2.4; 2.5]	27003	2.9 [2.8; 2.9]	5039	3.7 [3.6; 3.7]	5560	3.4 [3.3; 3.5]	$< 10^{-3}$
Private hospital	125[2]	3041	2.5 [2.4; 2.6]	6500	2.9 [2.8; 3.0]	1359	3.6 [3.5; 3.7]	1621	3.4 [3.3; 3.5]	$< 10^{-3}$
Public hospital	235[2]	2723	3.3 [3.2; 3.4]	4849	3.8 [3.7; 3.9]	940	5.1 [4.7; 5.9]	795	4.2 [3.8; 4.7]	$< 10^{-3}$
Average density of GE (GE/100000iHbts)										
Low	127[2]	4643	2.4 [2.3; 2.5]	8419	2.9 [2.8; 2.9]	1519	3.9 [3.8; 4.1]	1800	3.4 [3.3; 3.5]	$< 10^{-3}$
Average	108[2]	3245	2.5 [2.4; 2.5]	4314	2.9 [2.8; 2.9]	810	4.1 [3.8; 4.4]	781	3.6 [3.4; 3.9]	$< 10^{-3}$
High	467[2]	13621	2.6 [2.6; 2.7]	25619	3.0 [3.0; 3.1]	5009	3.8 [3.7; 3.9]	5395	3.5 [3.4; 3.6]	$< 10^{-3}$
Annual Nb of Colo locations										
1 location	483[2]	14437	2.6 [2.6; 2.7]	24851	3.0 [3.0; 3.1]	4763	3.8 [3.7; 3.9]	5160	3.5 [3.4; 3.6]	$< 10^{-3}$
≥ 2 locations	153[2]	7072	2.4 [2.4; 2.5]	13501	2.9 [2.9; 3.0]	2575	4.0 [3.9; 4.1]	2816	3.5 [3.4; 3.7]	$< 10^{-3}$
Residence of the patient										
Colonoscopy's supply municipality	338[2]	4947	2.5 [2.4; 2.5]	7775	2.9 [2.9; 3.0]	1502	3.9 [3.7; 4.1]	1530	3.5 [3.3; 3.6]	$< 10^{-3}$
Other municipality in Colonoscopy's supply department	480[2]	13259	2.5 [2.5; 2.6]	23754	3.0 [3.0; 3.1]	4401	3.9 [3.8; 4.0]	4982	3.5 [3.4; 3.6]	$< 10^{-3}$
Other departments in IDF	419[2]	3303	2.7 [2.6; 2.8]	6823	2.9[2.9; 3.0]	1435	3.8 [3.6; 4.0]	1464	3.6 [3.5; 3.8]	$< 10^{-3}$
Age (in yr) of the patients										
50-54	485[2]	4995	2.7 [2.6; 2.8]	8018	3.1 [3.0; 3.2]	1695	4.1 [3.9; 4.2]	1616	3.8 [3.7; 4.0]	$< 10^{-3}$
55-59	452[2]	4669	2.6 [2.5; 2.7]	7355	3.1 [3.0; 3.1]	1446	3.9 [3.7; 4.1]	1560	3.6 [3.4; 3.7]	$< 10^{-3}$
60-64	466[2]	4889	2.5 [2.4; 2.6]	7851	3.0 [2.9; 3.0]	1478	3.7 [3.5; 3.9]	1531	3.5 [3.4; 3.7]	$< 10^{-3}$
65-69	464[2]	3766	2.5 [2.4; 2.5]	8511	2.9 [2.8; 2.9]	1403	3.8 [3.6; 4.0]	1590	3.3 [3.1; 3.5]	$< 10^{-3}$
≥ 70	431[2]	3190	2.4 [2.3; 2.5]	6617	2.9 [2.8; 3.0]	1316	3.7 [3.5; 3.8]	1679	3.4 [3.3; 3.6]	$< 10^{-3}$

[1](Prob > F) ANOVA.

[2]This is the number of gastroenterologists (GEs) having performed a colonoscopy over the guaiac fecal occult blood test period, the same GE can be present in all of the modalities, for example the same GE having performed colonoscopies at 3 different sites corresponding to each of the density zones (low average high).

95%CI: 95% confidence interval; Colo: Screening colonoscopy; FIT: Fecal immunochemical test; GE: Gastroenterologist; gFOBT: Guaiac fecal occult blood test; iHbts: Inhabitants; IDF: Ile-De-France; Nb: Number.

The high proportion of incomplete colonoscopies due to insufficient preparation should alert to the need to set up a specific preparation protocol for screening colonoscopy. To date, it is impossible to evaluate with relevance the preparation of a colonoscopy in outpatients, who are not hospitalized at the time of the preparation. Similarly, there is no standard preparation scheme imposed in the French

Table 5 Proportion of colonoscopies performed beyond 7 mo and proportion of serious adverse events, according to the characteristics of the cohort of gastroenterologists who performed at least one colonoscopy in each of the three periods (guaiac fecal occult blood test, fecal immunochemical test, STOP-fecal immunochemical test, and COVID)

Characteristics of the cohort of gastroenterologists	Proportion of colonoscopies performed beyond 7 mo by period					Proportion of serious adverse events by period				
	gFOBT	FIT	STOP-FIT	COVID		gFOBT	FIT	STOP-FIT	COVID	
	Nb of Colo (% > 7 mo)	Nb of Colo (% > 7 mo)	Nb of Colo (% > 7mo)	Nb of Colo (% > 7 mo)	P^1	%EI	%EI		%EI	P^1
Overall	21509 (5.3)	38352 (6.2)	7342 (11.3)	7995 (9.2)	$< 10^{-3}$	0.3	0.3	0.3	0.2	0.10
Annual Nb of Colo										
1	304 (10.1)	150 (11.8)	38 (18.4)	51 (12.0)	$< 10^{-3}$	0.3	0	0	0	0.67
2-30	16819 (5.5)	15970 (6.9)	4887 (11.8)	4211 (9.4)	$< 10^{-3}$	0.3	0.4	0.3	0.1	0.09
31-100	4386 (4.1)	21137 (5.7)	2817 (10.6)	3733 (9.0)	$< 10^{-3}$	0.4	0.2	0.3	0.2	0.25
> 100		1095 (3.7)					0.2			
Place of Colo performance										
Clinic	15745 (4.8)	27003 (5.4)	5039 (10.4)	5560 (8.3)	$< 10^{-3}$	0.3	0.3	0.3	0.1	0.16
Private hospital	3041 (5.1)	6500 (5.9)	1359 (9.6)	1621 (9.5)	$< 10^{-3}$	0.3	0.2	0.2	0.2	0.71
Public hospital	2723 (8.5)	4849 (10.6)	940 (18.8)	795 (15.1)	$< 10^{-3}$	0.6	0.6	0.2	0.4	0.48
Average density of GE (GE/100000 iHbts)										
Low	4643 (5.1)	8419 (5.8)	1519 (11.5)	1800 (9.1)	$< 10^{-3}$	0.3	0.2	0.3	0.1	0.59
Average	3245 (4.9)	4314 (6.5)	810 (11.0)	781 (9.9)	$< 10^{-3}$	0.2	0.3	0.1	0.5	0.48
High	13621 (5.5)	25619 (6.2)	5009 (11.3)	5395 (9.2)	$< 10^{-3}$	0.4	0.3	0.3	0.1	0.04
Annual Nb of Colo locations										
1 location	14437 (5.6)	24851 (6.3)	4763 (10.6)	5160 (9.5)	$< 10^{-3}$	0.4	0.3	0.3	0.2	0.17
≥ 2 location	7072 (4.8)	13501 (6.0)	2575 (12.7)	2816 (8.7)	$< 10^{-3}$	0.2	0.2	0.2	0.1	0.80
Residence of the patient										
Colonoscopy's supply municipality	4947 (4.9)	7775 (5.9)	1502 (11.6)	1530 (8.0)	$< 10^{-3}$	0.2	0.3	0.3	0.0	0.18
Other municipality in Colonoscopy's supply department	13259 (5.1)	23754 (6.0)	4401 (11.0)	4982 (9.6)	$< 10^{-3}$	0.4	0.3	0.2	0.2	0.02
Other departments in IDF	3303 (6.6)	6823 (6.9)	1435 (12.1)	1464 (9.2)	$< 10^{-3}$	0.3	0.4	0.4	0.3	0.80
Age (in yr) of the patients										
50-54	4995 (6.7)	8018 (7.0)	1695 (12.2)	1616 (11.0)	$< 10^{-3}$	0.2	0.2	0.1	0.1	0.43
55-59	4669 (5.7)	7355 (6.6)	1446 (11.8)	1560 (9.9)	$< 10^{-3}$	0.3	0.3	0.4	0.2	0.79
60-64	4889 (4.7)	7851 (5.9)	1478 (10.3)	1531 (8.9)	$< 10^{-3}$	0.4	0.3	0.5	0.3	0.76
65-69	3766 (4.4)	8511 (5.5)	1403 (11.7)	1590 (8.2)	$< 10^{-3}$	0.4	0.3	0.3	0.1	0.20
≥ 70	3190 (4.6)	6617 (5.7)	1316 (10.6)	1679 (8.2)	$< 10^{-3}$	0.5	0.4	0.2	0.2	0.49

[1]Cochran *Q* test.

Colo: Screening colonoscopy; Colo+: Positive screening colonoscopy; FIT: Fecal immunochemical test; GE: Gastroenterologist; gFOBT: Guaiac fecal occult blood test; iHbts: Inhabitants; IDF: Ile-De-France; Nb: Number.

screening program, each GE proposing the method of his choice to the patient. However, although a non-superiority of a preparation scheme (Enema *vs* Oral preparation) was argued[21], studies admitted that a short time (1-6 h *vs* > 8 h) between the colic preparation and colonoscopy is associated with a

Table 6 Neoplasm detection rate at colonoscopy, according to the characteristics of the cohort of gastroenterologists who performed at least one colonoscopy in each of the three periods (guaiac fecal occult blood test, fecal immunochemical test, STOP-fecal immunochemical test, and COVID)

Characteristics of the cohort of gastroenterologists	Neoplasm detection rate at colonoscopy by period				
	gFOBT	FIT-1	STOP-FIT	COVID	
	Nb of Colo (% Colo+)	Nb of Colo (% Colo+)	Nb of Colo (% Colo+)	Nb of Colo (% Colo+)	*P*[1]
Overall	21509 (52.3)	38352 (65.0)	7342 (63.3)	7995 (60.1)	$< 10^{-3}$
Annual Nb of Colo					
1	304 (50.7)	150 (62.0)	38 (71.1)	51 (51.0)	0.02
2-30	16819 (53.6)	15970 (64.5)	4887 (63.7)	4211 (59.6)	$< 10^{-3}$
31-100	4386 (47.6)	21137 (65.8)	2817 (62.5)	3733 (61.8)	$< 10^{-3}$
> 100		1095 (58.6)	0	0	
Place of S-colo performance					
Clinic	15745 (52.4)	27 003 (64.9)	5039 (62.8)	5560 (60.1)	$< 10^{-3}$
Private hospital	3041 (54.3)	6500 (65.0)	1359 (64.8)	1621 (62.7)	$< 10^{-3}$
Public hospital	2723 (49.8)	4849 (65.6)	940 (63.7)	795 (59.5)	$< 10^{-3}$
Average Density of GE (GE/100000iHbts)					
Low	4643 (53.0)	8419 (64.5)	1519 (61.7)	1800 (58.4)	$< 10^{-3}$
Average	3245 (53.1)	4314 (64.1)	810 (65.2)	781 (61.1)	$< 10^{-3}$
High	13621 (51.9)	25619 (65.4)	5009 (63.4)	5395 (61.2)	$< 10^{-3}$
Annual Nb of S-colo locations					
1 location	14437 (52.8)	24851 (64.8)	4763 (63.0)	5160 (60.8)	$< 10^{-3}$
≥ 2 locations	7072 (51.3)	13501 (65.6)	2575 (63.8)	2816 (60.2)	$< 10^{-3}$
Residence of the patient					
Colonoscopy's supply municipality	4947 (53.4)	7775 (65.3)	1502 (61.1)	1530 (59.5)	$< 10^{-3}$
Other municipality in Colonoscopy's supply department	13259 (51.7)	23754 (65.0)	4401 (64.2)	4982 (61.2)	$< 10^{-3}$
Other departments in IDF	3303 (53.1)	6823 (64.9)	1435 (62.8)	1464 (59.6)	$< 10^{-3}$
Age (in yrs) of the patients					
50-54	4995 (44.9)	8018 (56.5)	1695 (55.2)	1616 (52.9)	$< 10^{-3}$
55-59	4669 (50.8)	7355 (63.1)	1446 (61.3)	1560 (58.9)	$< 10^{-3}$
60-64	4889 (54.2)	7851 (67.9)	1478 (65.5)	1531 (63.1)	$< 10^{-3}$
65-69	3766 (57.1)	8511 (68.9)	1403 (68.3)	1590 (65.3)	$< 10^{-3}$
≥ 70	3190 (57.5)	6617 (69.2)	1316 (67.9)	1679 (62.8)	$< 10^{-3}$

[1]Cochran *Q* test.

Colo: Screening colonoscopy; Colo+: Positive screening colonoscopy; FIT: Fecal immunochemical test; GE: Gastroenterologist; gFOBT: Guaiac fecal occult blood test; iHbts: Inhabitants; IDF: Ile-De-France; Nb: Number.

better quality of colonic preparation[26].

Compared to gFOBT, the high proportion of 2nd colonoscopies over the FIT period would confirm the literature on the performance of FIT in screening for precancerous lesions[27], which most often only require endoscopic resection. However, in addition to a high proportion of obstructive lesions, the proportion of severe cancers was significantly higher over the gFOBT period.

Several study results converge on a link between the long delay in access to colonoscopy and the CRC risk. Forbes *et al*[28] propose that wherever possible, colonoscopy should not be delayed beyond 6 mo of positive fecal testing as an aspirational target (with 9 mo as an upper limit). In the Kaiser Permanente (California) health plan members, the risk of CRC was increased by about 40% for any colonoscopy

Table 7 Multivariate analysis of the relationship between each dependent variable (binary variables 0/1: Screening colonoscopy performed beyond 7-mo; Serious adverse events, Yield of neoplasm at screening colonoscopy) and the predictive factors

Characteristics of the cohort of gastroenterologists	Colo performed beyond a 7-mo risk analysis		Serious adverse events risk analysis		Neoplasms risk analysis	
	OR_a, 95%CI	P^1	OR_a, 95%CI	P^1	OR_a, 95%CI	P^1
Annual Nb of Colo (Ref: 1 Colo)						
2-30	0.7 [0.6; 1.0]	0.002	2.5 [0.3; 18.2]	0.37	0.9 [0.7; 1.1]	0.41
> 30	0.7 [0.3; 0.9]	0.008	2.9 [0.9; 23.0]	0.05	0.8 [0.6; 1.1]	0.32
Place of S-colo performance (Ref: Clinic)						
Private hospital	1.2 [0.9; 1.6]	0.18	0.7 [0.3; 1.8]	0.47	1.1 [0.9; 1.3]	0.41
Public hospital	2.1 [1.3; 3.6]	0.001	1.6 [0.3; 8.7]	0.60	1.1 [0.8; 1.4]	0.20
Density of GE (Ref: Low)						
Average	0.9 [0.8; 1.0]	0.05	1.2 [0.6; 2.2]	0.59	1.0 [0.9; 1.1]	0.76
High	1.0 [1.0; 1.2]	0.28	1.2 [0.6; 2.3]	0.65	0.9 [0.8; 1.0]	0.04
Annual Nb of S-colo locations (Ref: 1 location)						
≥ 2 locations	1.1 [0.8; 1.5]	0.11	1.6 [0.5; 4;4]	0.41	1.0 [0.8; 1.3]	0.84
Residence of the patient (Ref: Colonoscopy's supply municipality)						
Other municipality in Colonoscopy's supply department	1.0 [0.9; 1.0]	0.30	1.0 [0.5; 2.1]	0.97	1.0 [0.9; 1.0]	0.31
Other departments in IDF	1.2 [1.1; 1.3]	$< 10^{-3}$	1.2 [0.3; 5.2]	0.81	0.9 [0.8; 1.0]	0.13
Age (yrs) of the patients (Ref: 50-54 yr)						
55-59	0.9 [0.8; 1.0]	0.03	1.6 [1.0; 2.6]	0.04	1.3 [1.2; 1.4]	$< 10^{-3}$
60-64	0.8 [0.7; 0.9]	0.001	2.0 [1.2; 3.1]	0.006	1.6 [1.5; 1.6]	$< 10^{-3}$
65-69	0.7 [0.7; 0.8]	$< 10^{-3}$	1.9 [1.2; 3.0]	0.01	1.7 [1.6; 1.8]	$< 10^{-3}$
≥ 70	0.7 [0.6; 0.8]	0.003	2.1 [1.3; 3.4]	0.002	1.7 [1.6; 1.8]	$< 10^{-3}$
Period (Ref.: gFOBT)						
FIT	1.2 [1.1; 1.2]	$< 10^{-3}$	0.8 [0.4; 1.5]	0.11	1.6 [1.5; 1.7]	$< 10^{-3}$
STOP-FIT	2.4 [2.1; 2.6]	$< 10^{-3}$	0.8 [0.5; 1.3]	0.27	1.3 [1.1; 1.5]	$< 10^{-3}$
COVID	2.0 [1.8; 2.2]	$< 10^{-3}$	0.5 [0.3; 0.9]	0.02	1.1 [1.0; 1.3]	0.08

[1]$P > |z|$.

95%CI: 95% confidence interval; Colo: Screening colonoscopy; FIT: Fecal immunochemical test; GE: Gastroenterologist; gFOBT: Guaiac fecal occult blood test; IDF: Ile-De-France; OR_a: Adjusted odds-ratio.

performed after a waiting period of 7-12 mo[17]. A recent meta-analysis shows that the risk of colorectal cancer is increased by 42%, and that the risk of cancer at an advanced stage was multiplied by 2 or even more, when colonoscopy was performed more than 6 mo after a positive test[29]. In this study, the time to access colonoscopy as well as its lengthening, induced first by the change of the test and then by the health crisis, had no impact in terms of the CRC severity, probably because of the discriminatory approach prioritizing patients with already existing symptoms. As a reminder, the French Society of Digestive Endoscopy had made, in mid-April 2020, the specific recommendation to postpone by 6 wk any colonoscopy following a positive screening test result, if there was no clinical nor biological sign of CCR[30]. In addition, since FIT was introduced in 2015 in a population screened biannually with gFOBT, the severe CRC screened by FIT are likely to be those not detected at an early stage by gFOBT. This hypothesis is confirmed by the drop in the colonoscopy detection rate and by the proportion of severe CRC over STOP-FIT and COVID periods.

To celebrate the tenth anniversary of the first atlas of medical demography, the National Council of the Order of Physicians focused on the gradual transfer from liberal activity to salaried activity. The focus also mentioned the widening of territorial inequalities to the detriment of regions and departments already in difficulty in terms of medical density[31]. Although the number of GEs is unevenly distributed over the 1268 IDF municipalities, the density of GEs in the IDF region was well

above the range (4.2 to 4.9) of the national average observed in 2017[31].

Each GE participating in a CRCSP must perform at least 300 colonoscopies per year[21]. Despite the superiority of the regional offer compared to the national average, the annual number of colonoscopies per GE stays very disparate and below 300, especially for GEs in public hospitals. The main limitation of this study is the fact that it only gives an opinion on screening colonoscopies. Indeed, screening colonoscopies only represented 5.5% of all colonoscopies performed in France in 2012 (gFOBT-period) and about 10% in 2016 (FIT-period)[32]. Since the patient base of a GE is not limited to the population of the region of practice, several GEs in the IDF region could reach or exceed this recommended annual number, in particular GEs practicing in a private clinic. The other limit of the study would come from the fact that the measurements of the indicators cannot be generalized over the whole of France. Indeed, the density of gastroenterologists and the types of practice (clinical hospital, *etc.*) may vary from one municipality (or department or region) to another. Only access to databases for the reimbursement of colonoscopy procedures could allow the exhaustive evaluation of such a quality indicator.

CONCLUSION

Although GEs are unevenly distributed over the municipalities of the IDF region, the supply of colonoscopies has remained almost constant between 2010 and 2020. The increase in colonoscopy requests induced by the change of the test kit has led to an increase in the average annual number of colonoscopies performed by GEs at the start of the FIT period. This very disparate annual average number between GEs fell over the STOP-FIT and COVID periods, due to the decrease in demand induced by the shutdown of the test kit market and the COVID-19 health crisis. The definition of a reference time and the obligation to respect it by all GEs would effectively reduce the time to access screening colonoscopy in France. The increase in the time to colonoscopy between the first and the second period was attributable to the introduction of the FIT, its increase after the second period was probably attributable to the malfunction of the program due to the slowdown of the kit market and the COVID-19 health crisis. Regardless of the characteristics of the GEs, the colonoscopy detection rate dropped significantly between the STOP-FIT and COVID periods, while the proportions of SAEs remained unchanged. However, the time to colonoscopy as well as its lengthening induced by the constraints had no impact in terms of CRC severity, probably because of a discriminatory approach prioritizing patients with existing symptoms.

ARTICLE HIGHLIGHTS

Research background

The impact of the Screening program on controlling the colorectal cancer (CRC) morbidity and mortality has been proved. But since its complete roll-out in 2009, the French population-based colorectal cancer screening program (CRCSP) experienced 3 major constraints [use of a less efficient Guaiac-test (gFOBT), Stopping the supply of Faecal-Immunochemical-Test kits (FIT), Suspension of the program due to the coronavirus disease 2019 (COVID-19)] affecting its effectiveness.

Research motivation

At this time when all the spotlights are focused on the impact of the health crisis linked to COVID-19, our motivation was to warn of the continued deterioration in the quality of screening colonoscopies in France.

Research objectives

To describe the impact of the constraints in terms of changes to the quality of screening colonoscopies.

Research methods

This retrospective cohort study included screening colonoscopies performed by the gastroenterologists between January 2010 and December 2020 in people aged 50-74 Living in Ile-de-France (France). The changes to the quality of screening colonoscopy (proportion of colonoscopies performed beyond 7 mo, Frequency of serious adverse events and the colonoscopy detection rate) were described in a cohort of Gastroenterologists who performed at least one colonoscopy over each of the four periods defined according to the chronology of the constraints [gFOBT: Normal progress of the CRCSP using gFOBT (2010-2014); FIT: Normal progress of the CRCSP using FIT(2015-2018); STOP-FIT: Year (2019) during which the CRCSP experienced the cessation of the supply of test kits; COVID: program suspension due to the COVID-19 health crisis (2020)]. The link between each dependent variable (Colo_7 mo; SAE Occurrence, Neoplasm detection rate) and the predictive factors was analyzed in a two-level multivariate hierarchical model.

Research results

The retrospective cohort was made up of 533 gastroenterologists. These 533 gastroenterologists achieved 21509 screening colonoscopies over the gFOBT period, 38,352 over FIT, 7342 over STOP-FIT and 7995 over the COVID period. The frequency of serious adverse events did not change between periods (gFOBT: 0.3%; FIT: 0.3%; STOP-FIT: 0.3%, and COVID: 0.2%; P = 0.10). The risk of colonoscopies performed beyond 7 mo doubled between FIT [adjusted-odds-ratio (aOR): 1.2 (1.1; 1.2)] and STOP-FIT [aOR: 2.4 (2.1; 2.6)], then decreased by 40% between STOP-FIT and COVID [aOR: 2.0 (1.8; 2.2)]. Regardless of the period, this Colo_7 mo's risk was twice as high for screening colonoscopy performed in a public hospital [aOR: 2.1 (1.3; 3.6)] compared to screening-colonoscopy performed in a private clinic. The neoplasm detection, which increased by 60% between gFOBT and FIT [aOR: 1.6 (1.5; 1.7)], decreased by 40% between FIT and COVID [aOR: 1.1 (1.0; 1.3)].

Research conclusions

The study showed that the constraints likely affected the time-to-colonoscopy as well as the colonoscopy detection rate without impacting the occurrence of the serious adverse events, highlighting the need for a respectable reference time-to-colonoscopy in CRCSP.

Research perspectives

At the end of this study, we initially aim to develop, evaluate, and validate a standard form for collecting data from screening colonoscopies in France. In a second step, we will evaluate the impact of the patient's motivation by the attending physician on the time taken to perform the colonoscopy.

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FOOTNOTES

Author contributions: Koïvogui A, Vincelet C, Abihsera G, Ait-Hadad H, Delattre H, Le Trung T, and Bernoux A are the doctors in charge of coordinating the screening program in each department; Nicolet J is the medical director of the CRCDC-IDF; Koïvogui A conceptualized and designed the project; all doctors in charge of coordinating the screening program collected the field data; Koïvogui A, Vincelet C, and Abihsera G analyzed the data, interpreted the results, and drafted the manuscript; all the authors revised the manuscript, read, and approved the final version of this manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comprehensively evaluate the short outcome of small bowel obstruction: A novel medical-economic score system

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Abstract

BACKGROUND

Small bowel obstruction (SBO) still imposes a substantial burden on the health care system. Traditional evaluation systems for SBO outcomes only focus on a single element. The comprehensive evaluation of outcomes for patients with SBO remains poorly studied. Early intensive clinical care would effectively improve the short-term outcomes for SBO, however, the full spectrum of the potential risk status regarding the high complication-cost burden is undetermined.

AIM

We aim to construct a novel system for the evaluation of SBO outcomes and the identification of potential risk status.

METHODS

Patients who were diagnosed with SBO were enrolled and stratified into the simple SBO (SiBO) group and the strangulated SBO (StBO) group. A principal component (PC) analysis was applied for data simplification and the extraction of patient characteristics, followed by separation of the high PC score group and the low PC score group. We identified independent risk status on admission *via* a binary logistic regression and then constructed predictive models for worsened management outcomes. Receiver operating characteristic curves were drawn, and the areas under the curve (AUCs) were calculated to assess the effectiveness of the predictive models.

RESULTS

Of the 281 patients, 45 patients (16.0%) were found to have StBO, whereas 236 patients (84.0%) had SiBO. Regarding standardized length of stay (LOS), total hospital cost and the presence of severe adverse events (SAEs), a novel principal component was extracted (PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE). In the multivariate analysis, risk statuses related to poor results for SiBO patients, including a low lymphocyte to monocyte ratio (OR = 0.656), radiological features of a lack of small bowel feces signs (OR = 0.316) and mural thickening (OR = 1.338), were identified as risk factors. For the StBO group, higher BUN levels (OR = 1.478) and lower lymphocytes levels (OR = 0.071) were observed. The AUCs of the predictive models for poor outcomes were 0.715 (95%CI: 0.635-0.795) and 0.874 (95%CI: 0.762-0.986) for SiBO and StBO stratification, respectively.

CONCLUSION

The novel PC indicator provided a comprehensive scoring system for evaluating SBO outcomes on the foundation of complication-cost burden. According to the relative risk factors, early tailored intervention would improve the short-term outcomes.

Key Words: Principal component analysis; Small bowel obstruction; Outcome evaluation system; Risk factors; Intensive clinical care; Radiomics

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Core Tip: A novel outcome indicator based on the standardized length of stay, total hospital cost and the presence of severe adverse events provided a comprehensive system for evaluating small bowel obstruction (SBO) outcomes. Furthermore, risk statuses associated with poor results were identified; specifically, for simple SBO patients, a low lymphocyte to monocyte ratio, as well as radiological features of a lack of small bowel feces signs and mural thickening, should be noticeable. For the strangulated SBO group, higher blood urea nitrogen levels and lower lymphocytes levels were recognized. Accordingly, early clinical intensive care was applicable for outcome improvement.

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INTRODUCTION

Small bowel obstructions (SBO) result in over 300000 hospitalizations *per* year in the United States[1]. With the increasing public health burden, the average cost for SBOs ranges from $30000-$38000 individually, and the total cost for SBOs is estimated to be approximately 9-11.4 billion dollars[2,3]. Recently, the short outcomes of SBO were evaluated by using in-hospital mortality, major complications and the length of hospital stay[3-6]. There is still lack of an integrative medical-economic system to evaluate the overall outcomes for SBO, even though previous studies have confirmed the relationship between worse outcomes and higher hospital costs[7,8]. Furthermore, the question of how to comprehensively evaluate outcomes for patients with SBO remains uncharted.

Principal component analysis (PCA) is commonly used for dimension reduction[9,10], linear correlation resolution and data simplification. By summarizing and maximizing the information encoding a set of outcome variables, a novel principal component for evaluating the clinical and economic effects on SBO is available. For SBO, patients' statuses on admission, including longer pain duration, acute kidney injury and malnutrition, were found to be closely correlated with severe adverse events (SAEs), based on previous studies[3,5,7,11]. However, the risk factors for the integrative scoring system, including clinical and economic adverse events, have not been extensively evaluated. The method of how to fully evaluate the potential risk status regarding the high complication-cost burden is urgently needed.

As an urgent life-threatening problem, the physical status of strangulated SBO is considerably deteriorating[12-14]. To control this confounding factor[15,16] and to further identify the risk admission status, we divided patients into a simple bowel obstruction group and a strangulated bowel obstruction group for the stratification analysis. We also constructed a novel indicator combining standardized SAEs, length of stay (LOS) and total hospital cost for defining outcomes of SBO. Furthermore, we

established a representative model to distinguish high-risk statuses for both the simple small bowel obstruction (SiBO) and strangulated small bowel obstruction (StBO) groups to guide clinical intensive care for SBO.

MATERIALS AND METHODS

Patient population

From October 2016 to February 2021, 479 patients diagnosed with intestinal obstructions at Fujian Medical University Union Hospital were included in the study. After excluding 180 cases with large bowel obstructions, 4 cases with missing computed tomography (CT) images and 13 cases with incomplete clinical data, 281 patients were recruited for the final study (shown in Figure 1). The following stratification was made according to the pathological confirmation of intestinal ischemia: A simple bowel obstruction (SiBO, n = 236) group and a strangulated bowel obstruction (StBO, n = 45) group. For patients without acute peritonitis, conservative treatment was applied. Once patients with highly suspect of bowel ischemia or failure to conservative treatment, laparoscopy as well as laparotomy was adopted for SBO patients according to different intrabdominal pressures (shown in Table 1). The study protocol was approved by the Institutional Review Board of Fujian Medical University Union Hospital (Approval No. 2021YF005-02), and all of the patients provided written informed consent for the procedure.

CT findings

All of the patients with suspected SBO underwent CT scans before receiving treatment. The features of the CT scans that were recorded in this study were separated into mesenteric fluid, ascites, spiral signs, concentric circle signs, small bowel feces signs and edema of the bowel wall categories[17-20]. All of the CT scan images were cross-reviewed and evaluated by two senior general surgeons (Chen XQ and Zhang JR, and both surgeons had abundant experience in abdominal emergency surgery. The definitions of CT characteristics are shown in Supplementary Figure 1 and supplied in Supplementary Table 1[21-25].

Clinical characteristics and laboratory tests

Baseline demographics consisted of sex, age, body mass index (BMI), comorbidity, temperature, pain duration and history of abdominal pain. Biochemical parameters, including white blood cell count, neutrophil percentage, lymphocyte concentration, monocyte concentration, hemoglobin concentration, platelet concentration, albumin, alanine aminotransferase, aspartate aminotransferase (AST), calcium concentration, chloride concentration, potassium concentration, sodium concentration, blood urea nitrogen (BUN), serum creatinine, glucose, prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer (DDI) and fibrinogen, were collected within 24 h of admission. Combinations of inflammatory parameters, such as the neutrophil to lymphocyte ratio and lymphocyte to monocyte ratio (LMR), were calculated and recorded accordingly.

Outcome definition

Posttreatment outcomes were both clinically and economically evaluated.

Postoperative complications were defined as any deviation from the normal postoperative course during the index admission for SBO treatment, which was guided by the European Perioperative Clinical Outcome definitions[7,26]. The severity of complications was graded according to the Clavien-Dindo (CD) system[27], which is a validated classification system that categorizes complication severity based on the level of required treatment. Grade I was defined as complications without the need for pharmacological treatment or surgical, endoscopic and radiological interventions, as well as only minor interventions such as vomiting; grade II was defined as complications requiring pharmacological or other treatments, such as blood transfusions and total parenteral nutrition; grade III was defined as complications requiring surgical interventions or other interventional treatments; grade IV was defined as life-threatening complications, including central nervous system, cardiac and pulmonary complications, as well as renal failure and those interventions requiring intensive care unit (ICU) management; and grade V was defined as death. CD grade I to grade III were classified as non-SAE, and CD grade IV to grade V were classified as SAE.

The LOS was defined as the number of days from admission to discharge. Total hospital cost was defined as the total expenditure for medical resource utilization during hospitalizations, which included fees for operations (materials and occupancy of the operating room), medications, radiology, laboratory tests, microbiology tests, ward stay, ICU days, feeding and blood products[28].

PCA

PCA was used to achieve data simplification by expressing multivariate outcome indicators with fewer dimensions. With standardized LOS, total hospital cost and the presence of SAEs, a novel principal

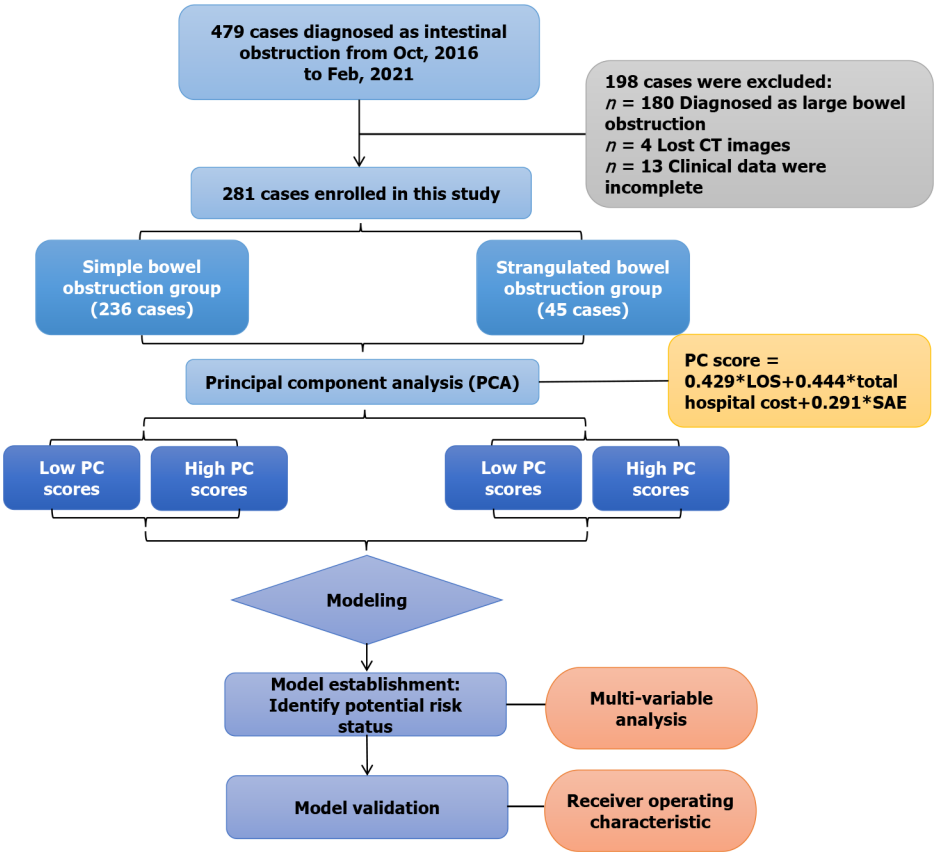
Table 1 Compared the clinical and laboratory characteristics of the patients with low or high principal component score

Characteristics	Simple obstruction (*n* = 236)		*P* value	Strangulated obstruction (*n* = 45)		*P* value
	Low PC score	High PC score		Low PC score	High PC score	
Baseline data						
Gender, *n* (%)			1.000[1]			0.421[2]
Male	117 (69.2%)	39 (69.6%)		18 (52.9%)	8 (72.7%)	
Female	52 (30.8%)	17 (30.4%)		16 (47.1%)	3 (27.3%)	
Age (yr)	60 (47, 69)	65 (53, 71)	0.081	63 (52.25, 70.00)	(61.0, 71.5)	0.321
BMI (kg/m^2)	20.70 (18.83, 22.98)	20.94 (18.21, 22.65)	0.196	20.20 (18.16, 22.00)	18.75 (17.72, 19.81)	0.228
Comorbidity, *n* (%)			0.245[1]			1.000[2]
None	128 (75.7%)	38 (67.9%)		27 (79.4%)	9 (81.8%)	
Yes	41 (24.3%)	18 (32.1%)		7 (20.6%)	2 (18.2%)	
Pain duration (d)	2 (1, 5)	6 (3, 12.5)	< 0.000	2.00 (1.00, 3.75)	2.0 (1.0, 4.0)	0.989
History of abdominal operation, *n* (%)			0.471[1]			0.603[2]
None	43 (25.4%)	17 (30.4%)		11 (32.4%)	2 (18.2%)	
Yes	126 (74.6)	39 (69.6%)		23 (67.6%)	9 (81.8%)	
Temperature (degrees Celsius)	36.6 (36.5, 36.8)	36.6 (36.5, 36.8)	0.401	36.6 (36.5, 36.8)	36.60 (36.50, 36.75)	0.956
CT characteristics						
Mesenteric fluid (%)			0.430			0.985[2]
None	32 (18.9%)	8 (14.3%)		1 (2.9%)	1 (9.1%)	
Yes	137 (81.1%)	48 (85.7%)		33 (97.1%)	10 (90.9%)	
Ascites (%)			0.849			1.000[2]
None	58 (34.3%)	20 (35.7%)		4 (11.8%)	1 (9.1%)	
Yes	111 (65.7%)	36 (64.3%)		30 (88.2%)	10 (90.9%)	
Spiral signs (%)			0.612[2]			0.436[2]
None	151 (89.3%)	52 (92.9%)		22 (64.7%)	5 (45.5%)	
Yes	18 (10.7%)	4 (7.1%)		12 (35.3%)	6 (54.5%)	
Concentric circle sign (%)			0.132[2]			0.745[2]
None	164 (97.0%)	51 (91.1%)		31 (91.2%)	11 (100%)	
Yes	5 (3.0%)	5 (8.9%)		3 (8.8%)	0 (0%)	
Small bowel feces sign (%)			0.006			1.000[2]
None	70 (41.4%)	35 (62.5%)		17 (50.0%)	5 (45.5%)	
Yes	99 (58.6%)	21 (37.5%)		17 (50.0%)	5 (54.5%)	
Mural thickness (median)	3.28 (2.30, 3.75)	3.63 (2.97, 4.53)	0.002	3.51 (3.16, 4.12)	3.42 (2.67, 4.07)	0.634
Laboratory data						
WBC (10^9/L)	6.770 (4.89, 9.52)	7.345 (4.87, 11.18)	0.387	8.70 (5.89, 12.23)	6.83 (10.01, 18.25)	0.384
NE%	75.50 (65.9, 83.3)	77.45 (68.5, 84.03)	0.422	83.60 (69.05, 86.90)	77.50 (73.30, 90.25)	0.853
Lymphocyte (10^9/L)	1.01 (0.74, 1.42)	0.94 (0.64, 1.34)	0.240	0.96 (0.65, 1.34)	0.60 (0.42, 0.76)	0.020
Monocyte (10^9/L)	0.420 (0.30, 0.58)	0.565 (0.34, 0.73)	0.011	0.570 (0.407, 0.735)	0.540 (0.33, 0.760)	0.721
NLR (ratio)	4.650 (3.03, 8.07)	6.115 (3.74, 9.30)	0.159	7.750 (4.085, 12.922)	9.030 (4.990, 15.565)	0.491
LMR (ratio)	2.286 (1.67, 3.42)	1.591 (1.13, 2.84)	0.002	1.681 (2.131, 1.141)	1.482 (0.957, 1.933)	0.459

Hb (g/L)	128.0 (115, 142)	120.5 (108, 133)	0.016	131.0 (110.0, 137.7)	129.0 (120.5, 145.0)	0.587
PLT (10^9/L)	205.5 (162.50, 250.75)	250.5 (180.25, 307.25)	0.002	213 (163, 260)	180.0 (152.0, 242.5)	0.256
Albumin (g/L)	35.9 (32.30, 40.45)	36.1 (31.80, 39.45)	0.403	34.6 (31.7, 39.6)	37.1 (28.6, 42.0)	0.977
ALT (U/L)	16 (11, 24)	16 (11, 22)	0.727	15.00 (12.00, 21.75)	15.00 (13.25, 27.75)	0.612
AST (U/L)	20 (16, 26)	21 (17, 25.5)	0.619	19.50 (17.00, 23.75)	36.50 (20.75, 45.25)	0.022
Ca (mmol/L)	2.19 (2.04,2.32)	2.15 (2.02,2.26)	0.152	2.19 (2.09, 2.31)	2.05 (1.95, 2.20)	0.062
Cl (mmol/L)	102.30 (100.0, 104.1)	100.15 (96.85, 104.03)	0.015	100.85 (98.13, 103.85)	102.00 (101.35, 104.15)	0.296
K (mmol/L)	4.035 (3.78, 4.34)	3.985 (3.74, 4.43)	0.957	4.00 (3.56, 4.31)	4.36 (3.62, 5.07)	0.290
Na (mmol/L)	138.40 (136.68, 140.48)	138.15 (135.50, 141.23)	0.533	138.05 (134.13, 140.30)	135.60 (134.75, 137.75)	0.334
BUN (mmol/L)	5.5 (4.3, 7.2)	5.4 (3.68, 8.23)	0.872	6.45 (4.00, 8.57)	10.6 (7.3, 15.3)	0.002
Glu (mmol/L)	6.78 (5.30, 8.67)	6.59 (5.16, 9.51)	0.515	8.165 (6.963, 9.300)	8.66 (6.78, 11.04)	0.428
PT (s)	13.6 (13.1, 14.3)	13.6 (13.28, 14.43)	0.825	13.45 (12.90, 13.90)	15.40 (14.20, 16.65)	0.004
APTT (s)	35.6 (33.3, 38.3)	36.1 (34.0, 40.9)	0.184	35.15 (32.18, 37.10)	41.6 (36.1, 45.0)	0.012
DDI (mg/L)	1.45 (0.71, 2.52)	1.94 (0.79, 4.67)	0.151	1.64 (0.88, 3.50)	5.75 (2.39, 6.72)	0.024
Fib (g/L)	3.49 (2.92, 4.37)	3.78 (3.25, 4.59)	0.150	3.69 (2.71, 4.60)	3.89 (3.17, 4.82)	0.548
Creatinine (umol/L)	70.0 (56, 81)	70.5 (54.75, 88.00)	0.512	67 (57, 76)	93 (80, 147)	0.003
Management			< 0.000[2]			0.215[2]
Conservative treatment	155 (91.7%)	17 (30.4%)		1 (2.9%)	0 (0%)	
Laparoscopy	11 (6.5%)	8 (14.3%)		8 (23.5%)	0 (0%)	
Laparotomy	3 (1.8%)	31 (55.4%)		25 (73.5%)	11 (100%)	
CD, *n* (%)			< 0.000[2]			< 0.000[2]
Grade I	141 (83.4%)	20 (35.7%)		5 (14.7%)	0 (0%)	
Grade II	28 (16.6%)	32 (57.1%)		28 (82.4%)	2 (18.2%)	
Grade III	0 (0%)	1 (1.8%)		1 (2.9%)	0 (0%)	
Grade IV	0 (0%)	3 (5.4%)		0 (0%)	9 (81.8%)	
Grade V	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
SAE, *n* (%)			0.018[2]			< 0.000[2]
None	169 (100%)	53 (94.6%)		34 (100%)	2 (18.2%)	
Yes	0 (0%)	3 (5.4%)		0 (0%)	9 (81.8%)	
Fee (¥)	12070 (8830, 19935)	54322 (41370, 74623)	< 0.000	51828 (33575, 66954)	83553.0 (74146.0, 142409.5)	< 0.000
Length of stay (d)	5 (4, 8)	16 (13.75, 22.50)	< 0.000	14.00 (10.25, 17.00)	28.0 (18.5, 35.5)	< 0.000

[1]were compared using the χ^2 test.
[2]were adjusted *P* values or Fisher's exact test.
SiBO: Simple small bowel obstruction; StBO: Strangulated small bowel obstruction; PC score: Principle component score; BMI: Body mass index; WBC: White blood cell; NE%: Neutrophil percentage; NLR: Neutrophil to lymphocyte ratio; LMR: Lymphocyte to monocyte ratio; Hb: Hemoglobi; PLT: Platelet; ALT: Alanine aminotransferase, AST: Aspartate aminotransferase; Ca: Calcium; Cl: Chloride; K: Potassiun; Na: Sodium; BUN: Blood urea nitrogen; Glu: Glucose; PT: Prothrombin time; APTT: Activated partial thromboplastin time; DDI: D-dimer; Fib: Fibrinogen; CD: Clavien-Dindo; SAE: Severe adverse event.

component was extracted: PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE. Furthermore, the patient population was classified in the following manner according to the quartile PC score: The low PC score group (below the 75% quartile) and the high PC score group (in the upper 75% quartile). This analysis was performed in R V.4.1.3 (R Foundation for Statistical Programming, Vienna, Austria) by using the psych packages.



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Figure 1 Workflow of this study. CT: Computed tomography; PC: Principal component; LOS: Length of stay; SAE: Severe adverse event.

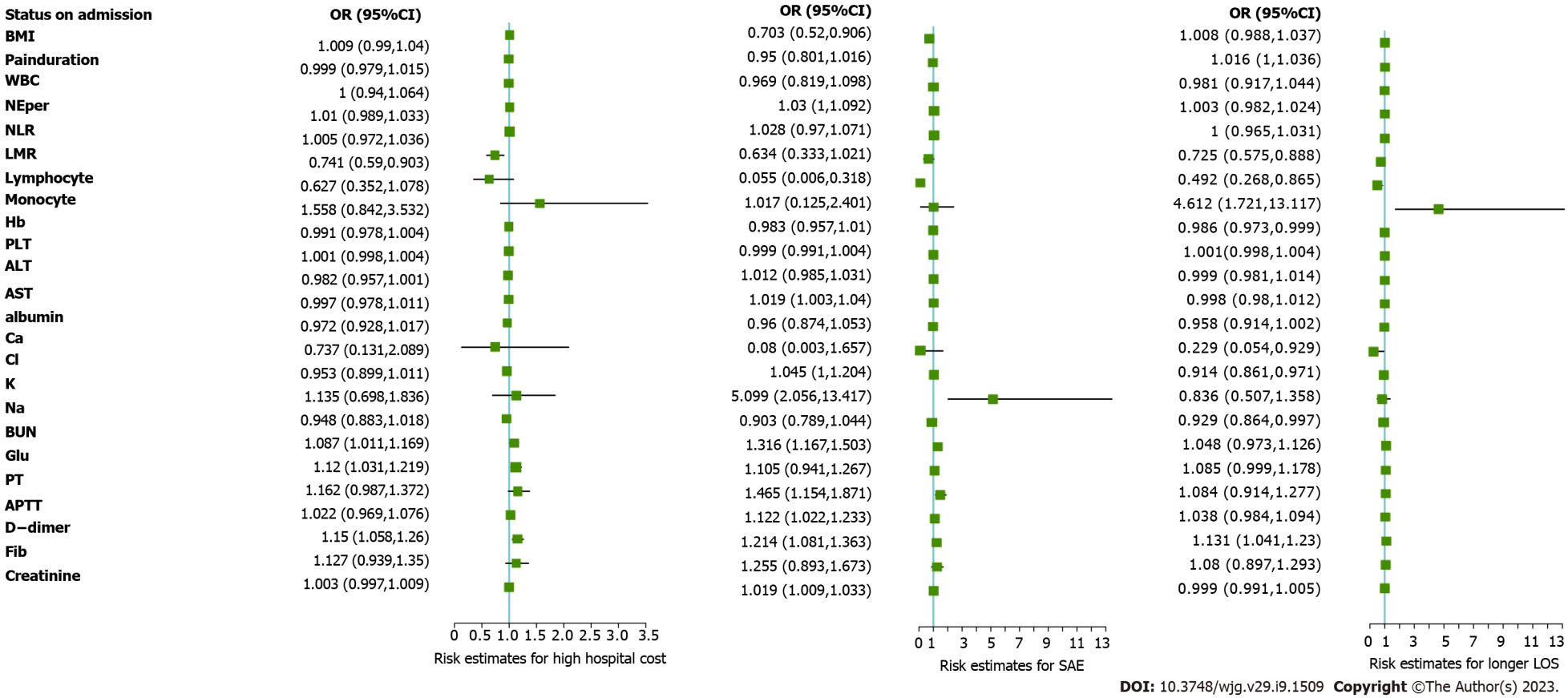
Statistical analysis

Categorical variables were compared by using the χ^2 test or Fisher's exact test between the two groups. Data are presented as the mean ± SD or median for continuous variables. Independent t tests or Kruskal-Wallis tests were applied according to the characteristics of the variables. The association of admission status with higher PC scores was evaluated by using univariate logistic regression and summarized with an odds ratio (OR) and 95% confidence interval (CI). After setting the variables with a significance level of $P < 0.05$ and variance inflation factors < 5, a multivariate logistic regression with "binomial" method was performed, and independent risk factors were determined. We extracted the following risk score formulas based on these independent risk factors: Risk score 1 (RS1) = [0.291 × (bowel wall thickness) - 1.150 × (small bowel feces sign) - 0.421 × (LMR)] and RS2 = [-2.632 × (lymphocyte concentration) + 0.391 × (BUN concentration)] for the SiBO group and StBO group, respectively. Receiver operating characteristic curves and the area under the curve were calculated to assess the accuracy of the models. All of the statistical analyses were performed in R Version.4.1.3. The statistical methods of this study were reviewed by Yin YR.

RESULTS

Outcome analysis

For 281 patients with SBO who were included in this study, posttreatment outcomes were evaluated by LOS, total hospital cost and the presence of SAEs. *Via* the univariate analysis, admission risk status, including lower LMR ($P = 0.005$), higher BUN concentration ($P = 0.022$), higher glucose concentration ($P = 0.007$) and higher DDI concentration ($P = 0.001$), was significantly associated with higher hospital costs. Patients with SAE had lower levels of lymphocyte concentration ($P = 0.003$), higher levels of AST ($P = 0.027$), higher levels of potassium ($P < 0.000$), higher levels of BUN ($P < 0.000$), higher levels of serum creatinine ($P < 0.000$) and coagulation and fibrinolysis disturbances, including longer PT ($P = 0.001$), APTT ($P = 0.012$) and higher levels of DDI ($P < 0.000$). Furthermore, at admission, lower LMR ($P = 0.003$), higher monocyte concentration ($P = 0.003$), lower hemoglobin concentration ($P = 0.038$), higher level of glucose ($P = 0.049$), higher level of DDI ($P = 0.004$) and abnormal electrolyte and metabolic changes, such as lower calcium concentration ($P = 0.042$), lower chloride concentration ($P = 0.003$) and lower sodium concentration ($P = 0.043$), were closely related to a longer LOS (Figure 2 and Supplemen-



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Figure 2 Risk factors for worse outcome of small bowel obstruction. Risk estimates for high hospital cost; Risk estimates for severe adverse event; Risk estimates for longer length of stay. OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; WBC: White blood cell; NE%: Neutrophil percentage; NLR: Neutrophil to lymphocyte ratio; LMR: Lymphocyte to monocyte ratio; Hb: Hemoglobin; PLT: Platelet, ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca: Calcium; Cl: Chloride; K: Potassiun; Na: Sodium; BUN: Blood urea nitrogen; Glu: Glucose; PT: Prothrombin time; APTT: Activated partial thromboplastin time; DDI: D-dimer; Fib: Fibrinogen; SAE: Severe adverse event; LOS: Length of stay.

tary Table 2).

PCA

After maximizing the possible information and variation of the above-mentioned outcome indicators, including total hospital cost, LOS and SAEs, data simplification was performed. *Via* PCA, one principal component was extracted (Supplementary Figure 2). The PC score was calculated according to weights given to each outcome indicator: PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE (Figure 1).

Of the 281 patients with SBO who were included in this study, 45 patients (16.0%) were found to have StBO, whereas 236 patients (84.0%) were found to have SiBO. The low PC score group (< 75% quartile) and high PC score group (> 75% quartile) were identified according to the quartile PC score. For both the SiBO and StBO groups, no significant difference was observed between the two PC score groups for sex, age, BMI, comorbidity status, temperature or history of abdominal operation (all *P* values > 0.05, Table 1). For patients with SiBO, a higher PC score was significantly related to longer pain duration (*P* < 0.000), higher monocyte concentration (*P* = 0.011), lower LMR (*P* = 0.002), lower hemoglobin concentration (*P* = 0.016), lower platelet count (*P* = 0.002) and low level of chloride (*P* = 0.015). Through the univariate analysis of radiological characteristics, we determined that a lack of small bowel feces signs and mural thickening were risk factors for a high PC score. In contrast, in the StBO group, low levels of lymphocytes (*P* = 0.020), high levels of AST (*P* = 0.022), high levels of BUN (*P* = 0.002) and coagulation and fibrinolysis disturbances, including abnormal DDI concentrations (*P* = 0.024), PTs (*P* = 0.004) and APTTs (*P* = 0.012), were significantly associated with higher PC scores. None of the risk radiological characteristics were observed in this stratification.

Univariate and multivariate analyses of risk statuses

Via the univariate analysis of the admission clinical-laboratory features, we determined potential risk status, including longer pain duration (*P* = 0.048), higher monocyte concentration (*P* = 0.003), lower LMR (*P* = 0.006), lower hemoglobin concentration (*P* = 0.033), lower platelet count (*P* = 0.036) and low level of chloride (*P* = 0.031), as well as radiological characteristics of mural thickening (*P* = 0.033) and lack of small bowel feces sign (*P* = 0.006), for high PC scores in the SiBO stratification. *Via* the multivariate analysis, independent risk factors consisting of radiological findings of small bowel feces sign (OR = 0.316), mural thickening (OR = 1.338) and LMR (OR = 0.656) were identified (all *P* values < 0.05, Table 2 and Figure 3). For StBO stratification, low levels of lymphocytes (*P* = 0.038), high levels of AST (*P* = 0.027), longer PTs (*P* = 0.015), high levels of BUN (*P* = 0.004) and creatinine (*P* = 0.022) seemed to be related to high PC scores. Finally, we found that only lymphocytes (OR = 0.071) and BUN (OR=1.478) were independent risk factors for high PC scores (all *P* values < 0.05, Table 2 and Figure 3).

Based on the regression coefficient for each factor, we calculated risk scores and built prediction models for worse outcomes: RS1 = [0.291 × (bowel wall thickness) - 1.150 × (small bowel feces sign) - 0.421 × (LMR)] for the SiBO group and RS2 = [-2.632 × (lymphocyte concentration) + 0.391 × (BUN concentration)] for the StBO group. Furthermore, receiver operating characteristic curves were drawn with areas under the curve of 0.715 (95%CI: 0.635-0.795) and 0.874 (95%CI: 0.762-0.986) for the SiBO and StBO stratifications, respectively (Figure 4).

DISCUSSION

Given that approximately 9-11.4 billion dollars are the costs *per* year in the United States, SBO still imposes a substantial burden on the health care system[2]. In contrast to the traditional evaluation systems that only focus on a single element[3-6], in this study, the standardized LOS, total hospital cost and the presence of SAEs were considered as integrative systems to evaluate the clinical-economic outcomes of SBO *via* PCA[9]. Previous studies have confirmed the close relationship between patients' statuses on admission (including longer pain duration, acute kidney injury and malnutrition) and adverse outcomes, which provides a potential target for improving outcomes[3,5,7,11]. Commonly, severe statuses, including severe inflammatory reactions, electrolyte disturbances and hemostatic abnormalities, tend to occur in strangulated bowel obstruction[22]. Following the formula that assigned weights to each component, we determined PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE; thus, the posttreatment outcome of SBO could be calculated and precisely evaluated (Figure 1).

For people with SiBO, only low LMR is observed, as radiological features (such as a lack of small bowel feces signs and mural thickening) were independent risk factors for high PC scores *via* the multivariate analysis. The area under the curve (AUC) of the predictive model based on the comprehensive scores for SiBO was 0.715 (95%CI: 0.635-0.795). As acute intestinal failure accompanies the obstructive bowel[26], when mechanical obstruction develops, the bowel lumen dilates along with the accumulation of air and intestinal fluid; thus, enteric stasis initiates bacterial proliferation with the intestinal gas produced by the fermentation of ingested food[22]. Conversely, when obstruction is incomplete or mild, the lasting bowel absorptive function can allow for fluid reabsorption across the bowel wall, thus leading to the small bowel feces sign as an independent protective factor for SBO[18,29]. Furthermore, progressive bowel dilation accompanied by compromised venous reflux increases intramural tension, which causes mural edema, secondary intestinal absorptive dysfunction and the loss of mucosal integrity (both functionally and physically)[22,30]. Similarly, as a potential effect on decreasing mural edema, the use of gastrografin challenge has been identified as the standardized management for SBO[31,32]. Moreover, in this study, the LMR was much lower in the high PC group, which may be due to the immune system becoming weakened as a result of the underlying malnutrition, as well as an excessive compensatory anti-inflammatory response[33-37].

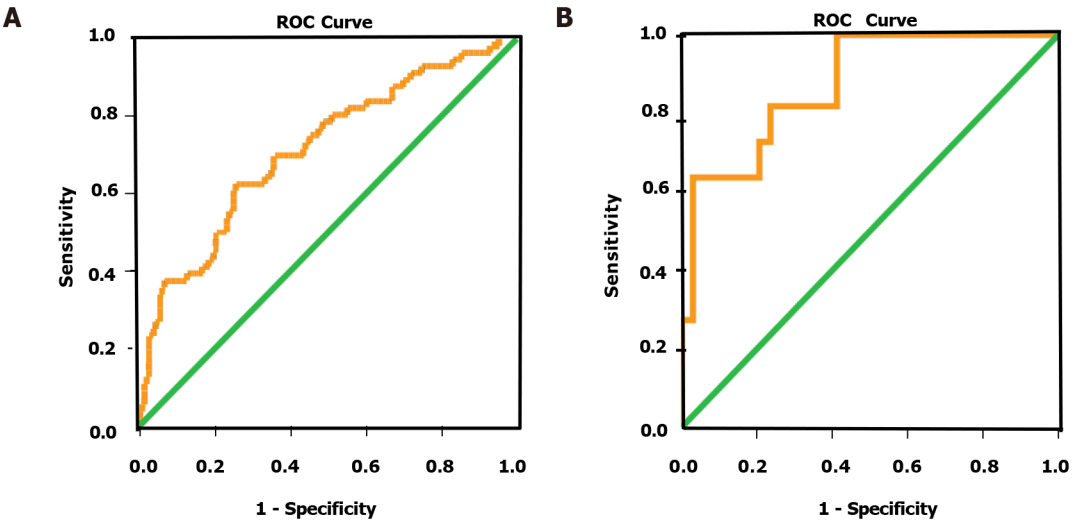
Table 2 Univariate and multivariate analyses of risk factors for high principal component score

Characteristics	Simple obstruction (*n* = 236)				Strangulated obstruction (*n* = 45)			
	Univariate		Multivariate		Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Pain duration (d)	1.019 (1.002, 1.041)	0.048[1]						
Small bowel feces sign (+)/(-)	0.424 (0.225, 0.783)	0.006[1]	0.316 (0.158, 0.612)	< 0.000[1]				
Mural thickening (cm)	2.119 (1.084, 4.375)	0.033[1]	1.338 (1.098, 1.664)	0.003[1]				
Lymphocyte (10^9/L)					0.097 (0.007, 0.665)	0.038[1]	0.071 (0.003, 0.539)	0.033[1]
Monocyte (10^9/L)	5.472 (1.809, 17.780)	0.003[1]						
LMR (ratio)	0.708 (0.541, 0.891)	0.006[1]	0.656 (0.496, 0.836)	0.001[1]				
Hb (g/L)	0.983 (0.969, 0.998)	0.033[1]						
PLT (10^9/L)	1.003 (1.001, 1.007)	0.036[1]						
AST (U/L)					1.075 (1.018, 1.156)	0.027[1]		
Cl (mmol/L)	0.931 (0.871, 0.993)	0.031[1]						
BUN (mmol/L)					1.383 (1.133, 1.786)	0.004[1]	1.478 (1.169, 2.061)	0.004[1]
PT (s)					1.568 (1.141, 2.418)	0.015[1]		
APTT (s)					1.109 (0.999, 1.264)	0.076		
DDI (mg/L)					1.196 (1.006, 1.513)	0.067		
Creatinine (umol/L)					1.034 (1.011, 1.071)	0.022[1]		

[1]indicates that the parameters have statistical difference ($P < 0.05$).

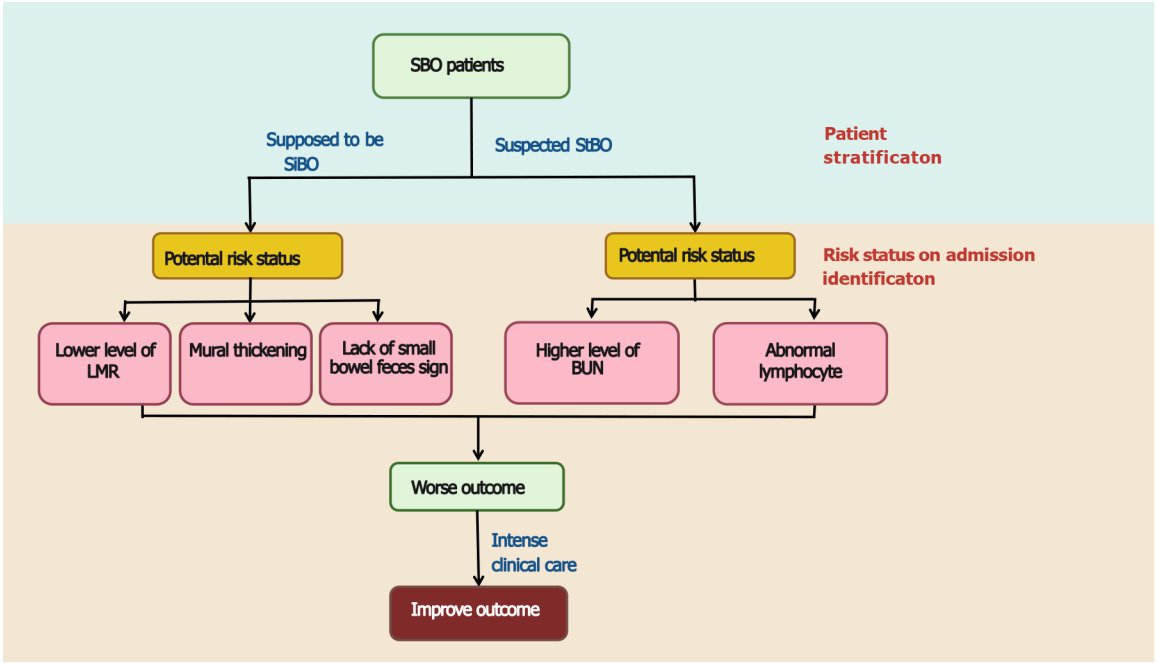
SiBO: Simple small bowel obstruction; StBO: Strangulated small bowel obstruction; OR: Odds ratio; CI: Confidence interval; LMR: Lymphocyte to monocyte ratio; Hb: Hemoglobin; PLT: Platelet; AST: Aspartate aminotransferase; Cl: Chloride; BUN: Blood urea nitrogen; PT: Prothrombin time; APTT: Activated partial thromboplastin time; DDI: D-dimer.

Once SiBO deteriorated into StBO, the risk factors were dynamically changed. None of the radiological characteristics were found to be related to the outcomes. In particular, coagulation and fibrinolysis disturbances (including abnormal DDI, PT and APTT), kidney injury (such as increasing BUN and creatinine levels) and relevant lymphocytes were confirmed as being risk factors. Finally, only BUN and lower lymphocyte counts were identified as being independent risk factors for high PC. Partially due to the impaired mucosal barriers[22,38], lactic acid from intestinal anaerobic glycolysis gradually accumulates, which adversely deteriorates renal function with increasing levels of BUN in the peripheral blood[39]. Similarly, it is difficult to correct conventional enteral interventions and intestinal mucosal malnutrition due to the weakened immune status[33,40], which may explain why a lower level



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Figure 3 Receiver operating characteristic curve for high principal component score prediction. The areas under the curve were 0.715 (95%CI: 0.635-0.795), 0.874 (95%CI: 0.762-0.986), respectively. A: Receiver operating characteristic curve of simple small bowel obstruction group for high principal component score prediction. B: Receiver operating characteristic curve of strangulated small bowel obstruction group for high principal component score prediction. ROC: Receiver operating characteristic.



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Figure 4 Proposal early clinical intensive care for small bowel obstruction patients on admission. SBO: Small bowel obstruction; SiBO: simple small bowel obstruction; StBO: Strangulated small bowel obstruction; LMR: Lymphocyte to monocyte ratio; BUN: Blood urea nitrogen.

of lymphocytes is a risk factor for poorer outcomes. The predictive model for StBO yielded an AUC of 0.874 (95%CI: 0.762-0.986), which provided an excellent differentiating ability.

There were a few limitations to the present study. Primarily, this was a retrospective study conducted in a single center. In addition, the sample size of the initial models was relatively small. However, in both group (SiBO or StBO) the patients evaluated were consecutively enrolled and this could reproduce a real-world situation. Adequately powered and well-designed studies are required to confirm these findings and to establish causality.

CONCLUSION

The novel PC indicator provided a comprehensive scoring system for evaluating SBO outcomes on the foundation of complication-cost burden. According to the relative risk factors, early tailored intervention would improve the short-term outcomes.

ARTICLE HIGHLIGHTS

Research background

Small bowel obstruction (SBO) still imposes a substantial burden on the health care system. Traditional evaluation systems for SBO outcomes only focus on a single element. There is still lack of an integrative medical-economic system to evaluate the overall outcomes for SBO. Moreover, patients' statuses on admission, including longer pain duration, acute kidney injury and malnutrition, were found to be closely correlated with severe adverse events (SAEs). However, the risk factors for the integrative scoring system, including clinical and economic adverse events, have not been extensively evaluated.

Research motivation

SBO still imposes a substantial burden on the health care system. Traditional evaluation systems for SBO outcomes only focus on a single element. The comprehensive evaluation of outcomes for patients with SBO remains poorly studied. Early intensive clinical care would effectively improve the short-term outcomes for SBO, however, the full spectrum of the potential risk status regarding the high complication-cost burden is undetermined.

Research objectives

In this study, we aim to construct a novel indicator combining standardized SAEs, length of stay (LOS) and total hospital cost for defining outcomes of SBO. Furthermore, we established a representative model for distinguishing high-risk statuses on admission for the simple SBO (SiBO) or strangulated SBO (StBO) groups. Given that SBO still imposes a substantial burden on the health care system, we believe our findings will provide a new insight for comprehensively evaluation outcomes of SBO as well as a guideline for early intervention.

Research methods

In this study, we evaluated posttreatment outcomes of SBO both clinically and economically. Principal component analysis (PCA) was used to achieve data simplification by expressing multivariate outcome indicators with fewer dimensions. By summarizing and maximizing the information encoding in standardized LOS, total hospital cost and the presence of SAEs, a novel principal component was extracted: PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE. Furthermore, the patient population was classified in the following manner according to the quartile PC score: The low PC score group (below the 75% quartile) and the high PC score group (in the upper 75% quartile).

Research results

In this study, a novel outcome indicator based on the standardized LOS, total hospital cost and the presence of SAEs provided a comprehensive system for evaluating SBO outcomes (PC score = 0.429 × LOS + 0.444 × total hospital cost + 0.291 × SAE). Furthermore, risk statuses associated with poor results were identified; specifically, for SiBO patients, a low LMR, as well as radiological features of a lack of small bowel feces signs and mural thickening, should be noticeable. For the StBO group, higher blood urea nitrogen levels and lower lymphocytes levels were recognized. Accordingly, early clinical intensive care was applicable for outcome improvement. In the future, adequately powered and well-designed studies are required to confirm these findings and to establish causality.

Research conclusions

In this study, PCA was innovatively used for dimension reduction, linear correlation resolution and data simplification. Furthermore, a novel comprehensive system for the evaluation of SBO outcomes was constructed and the potential risk status associated with poor results were identified.

Research perspectives

Large-scale and prospective studies are going to be designed to confirm these findings and to establish causality.

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FOOTNOTES

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SCIENTOMETRICS

Global trend and future landscape of intestinal microcirculation research from 2000 to 2021: A scientometric study

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Abstract

BACKGROUND

The intestinal microcirculation functions in food absorption and metabolic substance exchanges. Accumulating evidence indicates that intestinal microcirculatory dysfunction is a significant source of multiple gastrointestinal diseases. To date, there has not been a scientometric analysis of intestinal microcirculatory research.

AIM

To investigate the current status, development trends, and frontiers of intestinal microcirculatory research based on bibliometric analysis.

METHODS

VOSviewer and CiteSpace 6.1.R2 were used to identify the overall characteristics and knowledge map of intestinal microcirculatory research based on the core literature published from 2000 to 2021 in the Web of Science database. The characteristics of each article, country of origin, institution, journal, cocitations, and other information were analyzed and visualized.

RESULTS

There were 1364 publications enrolled in the bibliometric analysis, exhibiting an upward trend from 2000 to 2021 with increased participation worldwide. The

United States and Dalhousie University took the lead among countries and institutions, respectively. *Shock* was the most prolific journal, and *Nature Reviews Microbiology Clinical* had the most citations. The topical hotspots and frontiers in intestinal microcirculatory research were centered on the pathological processes of functional impairment of intestinal microvessels, diverse intestinal illnesses, and clinical treatment.

CONCLUSION

Our study highlights insights into trends of the published research on the intestinal microcirculation and offers serviceable guidance to researchers by summarizing the prolific areas in intestinal disease research to date.

Key Words: Intestinal microcirculation; Bibliometric analysis; CiteSpace; VOSviewer; Visualization

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Core Tip: This bibliometric analysis of the research directions and important literature related to the intestinal microcirculation over the last 22 years documents the current status, development trends, and frontiers of intestinal microcirculatory research and provides information that may guide future research efforts.

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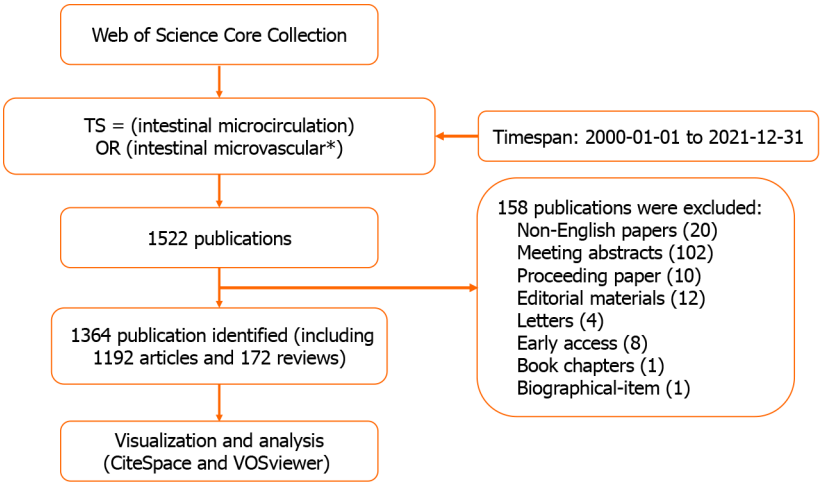
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INTRODUCTION

The intestines function in food absorption and metabolic substance exchange. Approximately 20%-25% of cardiac output is directed toward the digestive tract under physiological conditions. Among them, about 60%-80% flow to the submucosal and mucosal layers[1], providing the highly metabolically active epithelial, immune with nutrients and oxygen. Intestinal microvessels and lymph capillaries serve as an integrated system (so-called intestinal microcirculation) providing multiple bidirectional transport processes while defending the lumen against the threat of chemicals and bacteria. The intestinal microcirculation regulates a variety of metabolic and physiological processes involved in diseases such as shock, sepsis[2,3], gastrointestinal diseases[4], and diabetes mellitus[5].

Recent studies have shown that intestinal microcirculatory dysfunction is characterized by nutritive perfusion failure, inflammatory cell responses, surges in proinflammatory mediators, and breakdown of epithelial barrier function, as well as bacterial translocation and the development of systemic inflammatory responses[6-8]. Notably, there is widespread consensus that ischemic injury and severe microcirculatory dysfunction in the highly vascularized gut are significant sources of multiple organ dysfunction and even death[9,10]. Moreover, the intestinal microcirculation behaves as an isolated area in patients with postoperative abdominal sepsis[11], suggesting that the intestinal microcirculation does not always correlate with systemic hemodynamic variables (for example, blood pressure)[12] in gastrointestinal diseases. Therefore, it is rational to have a comprehensive scenario that depicting the functional status of intestinal microcirculation in discussing the specific issues. However, there have been few attempts to systematically assess the scientific findings and current networks in this field from a worldwide perspective.

Bibliometrics analyzes the quantitative relationships, distribution structure, and cocitation patterns of publications using mathematical and statistical methods, revealing the disciplinary development direction and research dynamics of related fields and illustrating the key paths and knowledge nodes of disciplinary field evolution[13]. This study provided a bibliometric analysis of the research process and important literature related to the intestinal microcirculation over the last 22 years to provide information for future research on the intestinal microcirculation.



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Figure 1 The flow chart for the search and selection strategy of the study.

MATERIALS AND METHODS

Search strategies

The literature data for this study were retrieved from Clarivate Analytics' Web of Science Core Collection (WOSCC). The key topic for retrieval was TS = (intestinal microcirculation) OR (intestinal microvascular*). The timespan was limited from January 1, 2000 to December 31, 2021. The data were obtained within one day to avoid any potential discrepancies due to daily updates of the database. Only English-language original articles and reviews were selected. Consequently, a total of 1364 publications, comprising 1192 articles and 172 reviews, were retrieved, and each literature record included relevant information such as title, author, keywords, abstract, year, organization, and citation. A summary of the search and selection methodology for the study can be found in Figure 1.

Data analysis and visualization

In this current study, CiteSpace 6.1.R2 (https://citespace.podia.com) was adopted to map cooperation networks (institutions) and document cocitation clustering, and keyword clustering. The set of parameters was as follows: The time slice was set to one year for articles published from 2000 to 2021, and the node types were "institution", "reference", and "keyword", with a g-index k value of 25. Different parameters were set following different node types, and the visualization map was drawn.

VOSviewer (1.6.18) (www.vosviewer.com) was used to identify and illustrate the co-occurring countries/regions, cocitation analysis of journals and references, and analysis of keyword co-occurrence. The screening condition and thresholds were as follows: The counting method was set as "Full counting" with a minimum number of 5 and a maximum of 1000 items.

RESULTS

Characteristics and trends of publications

The annual publication trend reflects the development level of intestinal microcirculatory research[14]. From 2000 to 2021, a total of 1364 intestinal microcirculation-related articles met the retrieval standard. Subsequently, we illustrated the article counts per year with a histogram. According to Figure 2, the annual number of relevant publications was rather consistent, with a mean of 65 publications, indicating sustained attention from 2000-2021. Although articles accounted for most of the literature, there was a considerable increase in reviews from 2019-2021, indicating a growing interest in the intestinal microcirculation.

Contributions by countries/regions and institutions

The number of papers published by research groups according to country/region and institution can reflect the distribution of research forces in the field of intestinal microcirculatory research. In Table 1, the top 10 countries and institutions were ranked based on the number of publications related to the intestinal microcirculation. With 420 publications accounting for 30.79% of the total, the United States was the top-producing nation, followed by Germany (231, 16.94%) and China (164, 12.02%). The co-occurrence map demonstrated that the United States attached great importance to cooperation and worked closely with Germany, England, Canada, and other European countries (Figure 3). In addition,

Table 1 The top 10 countries and institutions contributed to publications on intestinal microcirculation

Rank	Country	Count	Institution	Count
1	United States	420	Dalhousie Univ	41
2	Germany	231	Univ Szeged	35
3	China	164	Univ Amsterdam	33
4	Japan	113	Louisiana State Univ	31
5	England	77	Med Coll Wisconsin	29
6	Canada	69	Univ Louisville	29
7	Italy	67	Univ Munster	23
8	Netherlands	63	Univ Sao Paulo	23
9	France	51	China Agr Univ	20
10	Sweden	47	Lund Univ	20

Table 2 The top 10 journals and cited journals of intestinal microcirculation research

Rank	Journal	Count	Cited-journal	Count
1	*Shock*	53	*Nature Reviews Microbiology*	2930
2	*Journal of Surgical Research*	37	*Clinical Microbiology Reviews*	1533
3	*World Journal of Gastroenterology*	36	*Journal of Pathology*	1520
4	*American Journal of Physiology-Gastrointestinal and Liver Physiology*	34	*Critical Care Medicine*	1499
5	*Critical Care Medicine*	34	*Gastroenterology*	1372
6	*Clinical Hemorheology and Microcirculation*	27	*American Journal of Physiology-Gastrointestinal and Liver Physiology*	1175
7	*Microcirculation*	20	*World Journal of Gastroenterology*	1126
8	*Microvascular Research*	95	*Science*	968
9	*Plos One*	73	*Shock*	965
10	*American Journal of Physiology-Heart and Circulatory Physiology*	70	*Journal of Immunology*	937

colleges and universities were the major sources of intestinal microcirculatory research. Dalhousie University was identified as the most productive scientific institution, with the most papers (41), followed by the University of Szeged (35) and the University of Amsterdam (33). These findings highlighted useful information on prominent research teams and established collaborative ties.

Analysis of journals and cited journals

Table 2 lists the top 10 most prolific journals and most-cited journals. *Shock* (53) published the most papers about the intestinal microcirculation, followed by the *Journal of Pathology* (37), and the *World Journal of Gastroenterology* (36). *Nature Reviews Microbiology* had the highest number of local citations (2930 local citations) in the field, *Clinical Microbiology Reviews* was the second-cited journal (1533 local citations) and the *Journal of Pathology* (1520 local citations) was the third. Additionally, a dual-map overlay of journals with four colored pathways was established to reflect the disciplinary distribution of academic journals (Figure 4). Most clusters of citing and cited journals are located in medicine, clinical, molecular, biology, and immunology.

Analysis of co-cited references

The landmark literature and the rapid development of this field can be clarified through the cocitation analysis of relevant publications[15]. We then established the co-cited reference network map (Figure 5A), and through cluster analysis, similar references were categorized into knowledge units (Figure 5B). Additionally, the modularity value (Q value) and the mean silhouette value (S value) were used to evaluate the effect of the literature cocitation mapping.

With more than 5000 references cited in the last 22 years, the top 10 most cited articles about the intestinal microcirculation are listed in Table 3. (Binion DG, 1997), which had a citation count of 65, was

Table 3 Top 10 highly cited publications in intestinal microcirculation

Rank	Title	Citations	Journal	First author	Published year
1	Enhanced Leukocyte Binding by Intestinal Microvascular Endothelial Cells in Inflammatory Bowel Disease	65	*Gastroenterology*	David G. Binion	1997
2	Intestinal mucosal lesion in low-flow states	62	*Archives of Surgery*	Chu-Jeng Chiu	1970
3	The microcirculation is the motor of sepsis	49	*Critical care*	Can Ince	2005
4	Microvascular Blood Flow Is Altered in Patients with Sepsis	46	*American Journal of Respiratory and Critical Care Medicine*	Daniel De Backer	2002
5	How to evaluate the microcirculation: report of a round table conference	45	*Critical care*	Daniel De Backer	2007
6	Preparation of rat intestinal muscle and mucosa for quantitative microcirculatory studies	43	*Microcirculation research*	H.Glenn Bohlen	1976
7	Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock	42	*Critical care medicine*	Yasser Sakr	2004
8	Microcirculatory oxygenation and shunting in sepsis and shock	34	*Critical care medicine*	Can Ince	1999
9	Angiogenesis as a Novel Component of Inflammatory Bowel Disease Pathogenesis	32	*Gastroenterology*	Silvio Danese	2006
10	Ischemia–Reperfusion Injury of the Intestine and Protective Strategies Against Injury	32	*Digestive Diseases and Sciences*	Ismail Hameed Mallick	2004

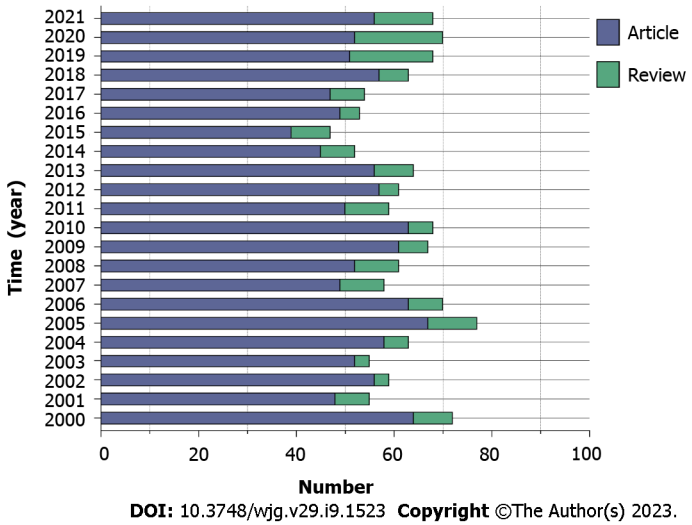
Table 4 Top 10 keywords in terms of frequency

Rank	Frequency	Centrality	Keywords
1	136	0.05	Expression
2	136	0.04	Nitric oxide
3	134	0.06	Blood flow
4	109	0.05	Rat
5	106	0.03	Injury
6	100	0.04	Microvascular endothelial cell
7	100	0.08	Inflammatory bowel disease
8	92	0.06	Microcirculation
9	85	0.08	Sepsis
10	85	0.05	Septic shock

the top-ranked article. (Chiu CJ, 1970), with 62 citations, and (Ince C, 2005), with 49 citations, followed. Moreover, 9 clusters were identified for mitochondrial respiration, sepsis, tissue oxygen tension, ischemia-reperfusion injury, hemorrhagic shock, endothelium, adenosine 5-triphosphate, gut, and no-reflow. The Q value of the clustering map was 0.9342, and the S value was 0.9579, confirming that the structured network was well mapped and that the clustering results were effective and reliable.

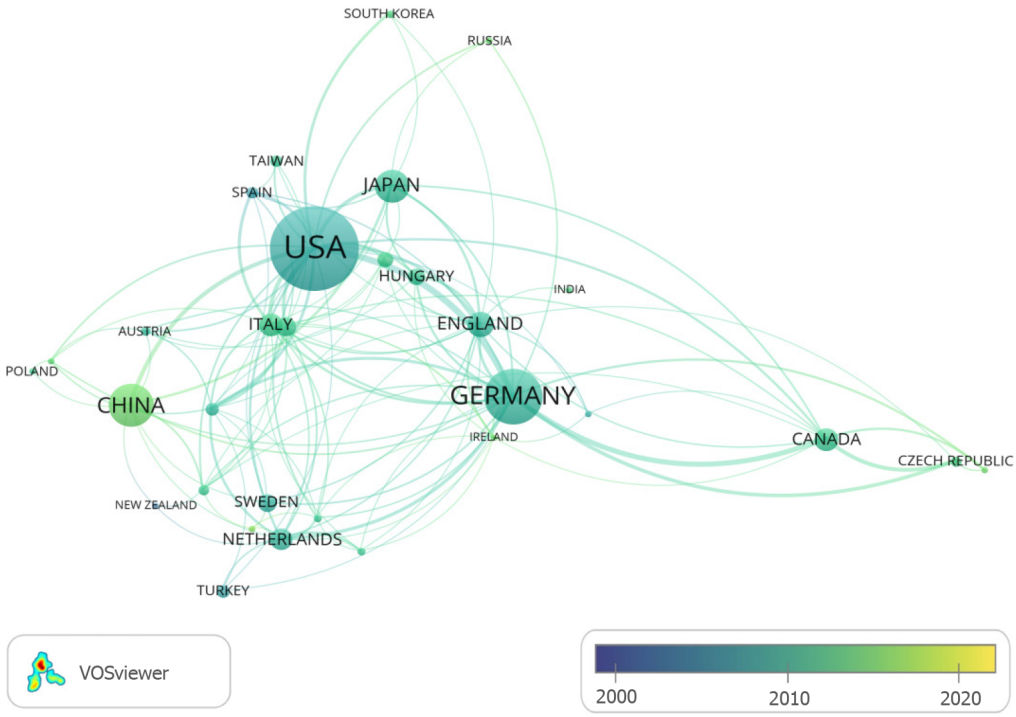
Analysis of keywords

Keywords refer to a high-level overview and refinement of the study topic and article content[16]. In terms of frequency, the top 10 keywords in the intestinal microcirculatory research from 2000 to 2021 were determined by creating a graphical map of keyword co-occurrence (Figure 6A and Table 4). The top keywords were "expression", "nitric oxide", "blood flow", "rat", "injury", "microvascular endothelial cell", "inflammatory bowel disease", "microcirculation", "sepsis" and "sepsis shock". Clustering analysis was carried out based on the above results and the following 10 clusters were identified (Figure 6B), which represented the key research areas. Specifically, the clusters "nitric oxide", "Shiga toxin" and "alkaline phosphatase" explored the mechanisms and pathological basis of damage to the intestinal microcirculation; the clusters "septic shock" and "inflammatory bowel disease" were diseases related to the intestinal microcirculation; the cluster "intravital microscopy" represented an effective measurement technique; and the clusters "cytokine therapy" and "negative pressure wound



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Figure 2 Distribution of articles published in the intestinal microcirculatory research from 2000 to 2021. The chart showed trends in annual publishing during the previous 22 years. Purple bars represent the number of articles related to intestinal microcirculation per year, while green bars represent the number of reviews.

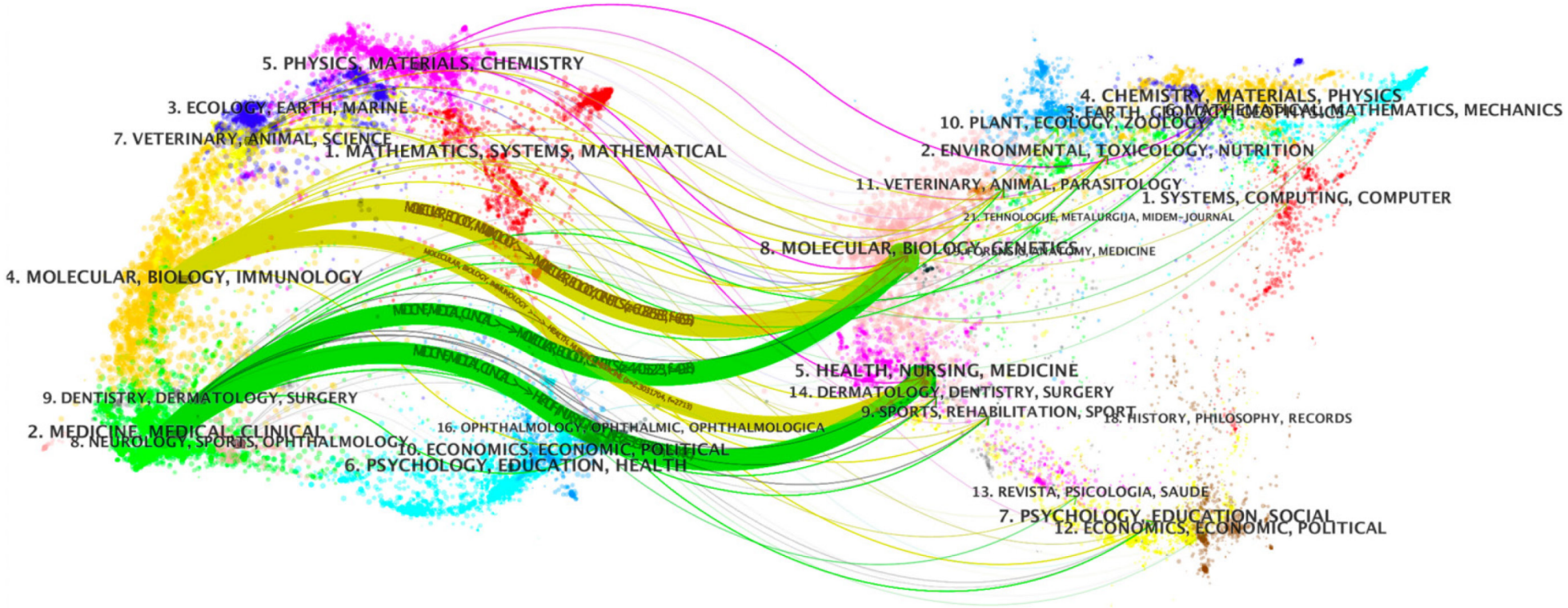


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Figure 3 The network of countries/regions engaged in the intestinal microcirculatory research. The collaborations were generated after a minimum of five publications per country. Of the 62 countries active in this field, 32 countries meet this criterion. The size of the node represents the number of posts, and the links between the nodes represent the connection or cooperation between the countries. The transition from blue to yellow in the color bar depicts the years 2000 to 2021.

therapy" involve effective and reliable countermeasures for intestinal microcirculatory dysfunction.

Burst keywords also highlight hotspots and developing trends; hence, the detection of keywords with the fastest increase in citations (citation bursts) denotes the emerging focus in dynamic domains[17]. Our analysis revealed the top 25 keywords for the strongest citation bursts from 2000 to 2021 (Figure 7). Among them, the highest burst strength (10.34) was found for "multiple organ failure" since 2000, and also the longest-lasting burst term was "endothelial growth factor" (2007-2019). From 2000 to 2005, the mechanism tended to be more actively researched based on the main keywords "neutrophil", "adhesion", "free radical", and "platelet-activating factor". Since 2006, researchers have begun to investigate the potential correlation between clinical gut illnesses and microcirculation, with the main



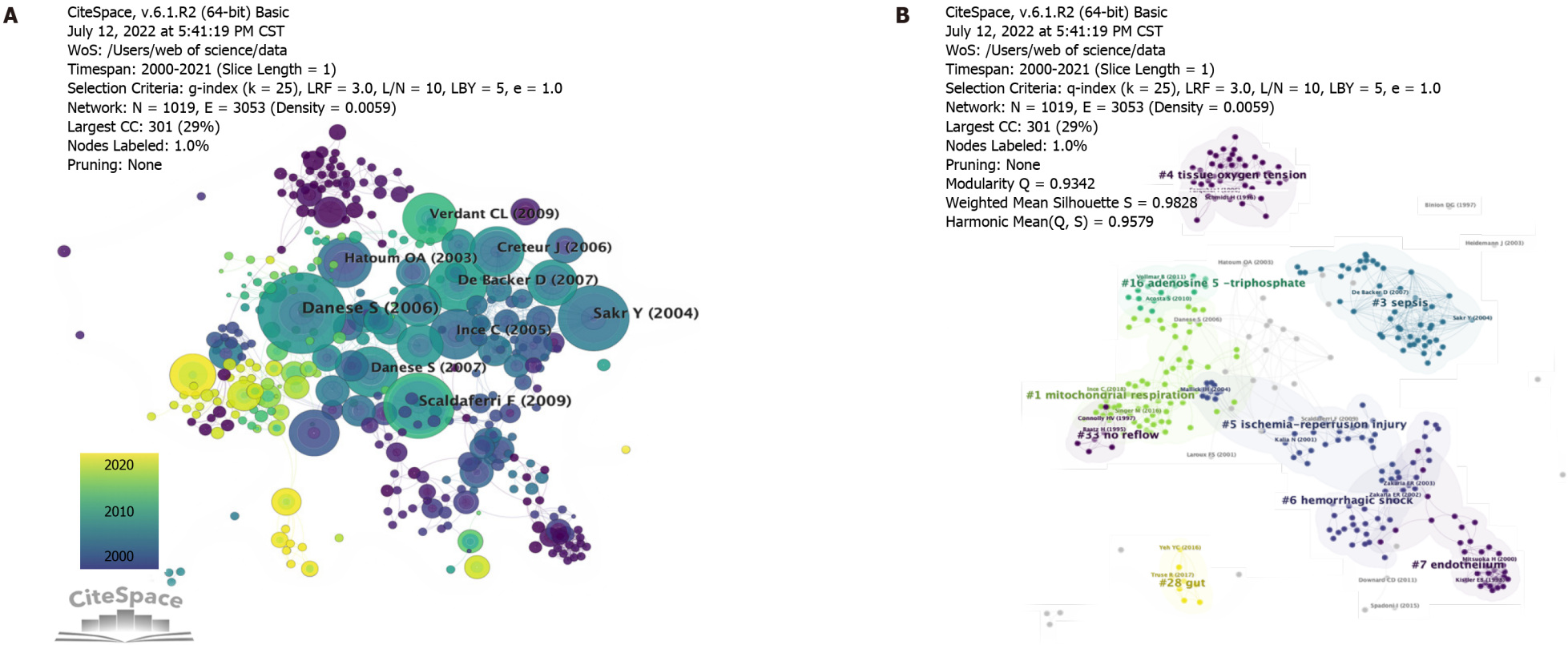
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Figure 4 The dual-map overlay of journals in the intestinal microcirculatory research. The left panel shows the map of citing journals while the right panel represents the map of the cited journals. The labels represent the scientific subject covered by the journals. Colored paths indicate the citation relationships, with the thicker lines representing the main pathways.

keywords being "ulcerative colitis", "Crohn's disease", and "necrotizing enterocolitis". In addition, the keyword for the most recent burst was "gut microbiota" (2019), suggesting it has been in the spotlight so far.

DISCUSSION

This study performed a scientometric analysis of publications on the intestinal microcirculation published from 2000 to 2021 using CiteSpace and VOSviewer. The findings provided insight into recent developments in global research collaborations, the most active journals, the core research areas, and emerging research areas.

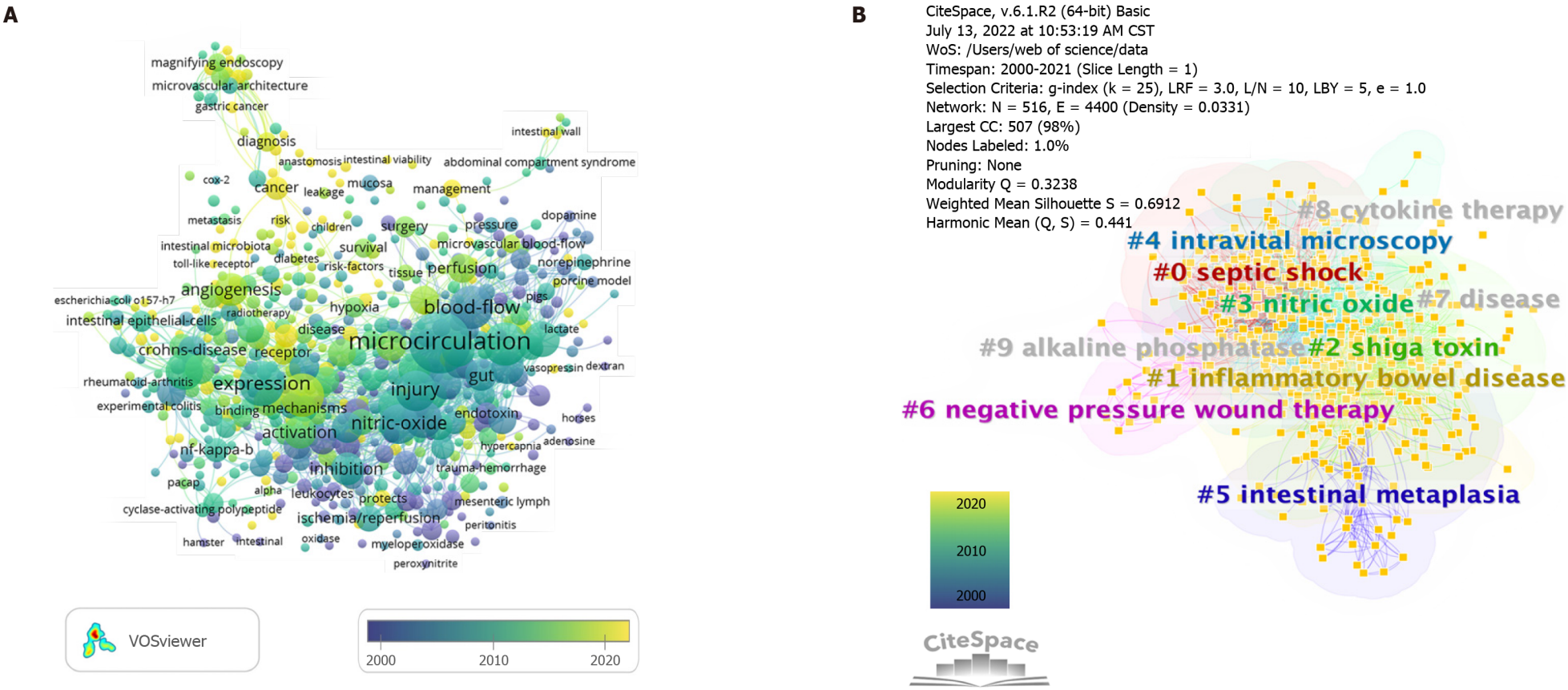


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Figure 5 The network map of co-cited references in the intestinal microcirculatory research. A: The network map of co-cited references. Nodes in the visualized network represent co-cited references and lines between nodes represent co-cited links; B: The network map of co-cited clusters. 9 clusters with diversified research themes were formed and illustrated in different colors. Silhouette = 0.9828. Modularity Q = 0.9342.

In total, 1364 publications about the intestinal microcirculation were extracted from WOSCC. Although the trend of annual publications from 2000 to 2021 reflected the continued interest of scholars in intestinal microcirculatory research, in comparison to research on the microcirculation of other tissues and organs, the annual number of papers on the intestinal microcirculation is relatively low, which may be associated with the technical and clinical challenges involved in the research. The United States and Germany are thriving hubs of intestinal microcirculatory research due to increased collaborations and strengthened citation links between several European countries, suggesting that a strong level of collaboration promotes academic influence.

Notably, journals focusing on clinical practice and published reviews and articles on the crucial role of the intestinal microcirculation in the progression of gastrointestinal diseases. Additionally, a dual-



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Figure 6 The network map of keywords in the intestinal microcirculatory research. A: The co-occurrence map of keywords in the intestinal microcirculatory research. The graphical mapping of terms was created when setting the minimum number of keyword occurrences to 5. Of the 6607 keywords in the field, 572 reached this threshold. Each node represents a keyword, and the sizes of the node denote the number of occurrences of the keywords map. The transition from blue to yellow in the color bar depicts the years 2000 to 2021; B: The clustering map of keywords in the intestinal microcirculatory research. 10 clusters with diversified themes were formed and illustrated in different colors. Colors represent clusters of the close-working network. Silhouette = 0.3238. Modularity Q = 0.6912.

map overlay of journals demonstrated that the research was focused on basic and clinical medicine, thus, multidisciplinary efforts are needed to support the development of the intestinal microcirculatory field. Co-cited references revealed that (Binion DG, 1997), with the highest frequency of citations, was a representative reference that laid the foundation for intestinal microcirculatory mechanisms in inflammatory bowel disease. Other landmark publications such as (Ince C, 2005) and (Daniel DB, 2002), outlined mechanisms of interaction between sepsis and microcirculation. In addition, (Daniel DB, 2007)

Top 25 keywords with the strongest citation bursts

Keywords	Year	Strength	Begin	End	2000-2021
Multiple organ failure	2000	10.34	**2000**	2005	
Neutrophil	2000	8.55	**2000**	2003	
Adhesion	2000	6.77	**2000**	2003	
Intestinal ischemia reperfusion	2000	6.4	**2000**	2004	
Tumor necrosis factor	2000	4.89	**2000**	2007	
Free radical	2000	4.85	**2000**	2005	
Microvascular permeability	2000	4.85	**2000**	2005	
Platelet activating factor	2000	4.58	**2000**	2005	
P selectin	2000	4.96	**2002**	2004	
Small intestine	2000	4.35	**2003**	2005	
Endothelial cell dysfunction	2000	4.68	**2004**	2005	
Ulcerative coliti	2000	5.3	**2006**	2011	
Severe sepsis	2000	6.31	**2007**	2012	
Endothelial growth factor	2000	4.38	**2007**	2019	
Crohns disease	2000	5.29	**2008**	2011	
Intestinal metaplasia	2000	4.63	**2008**	2012	
Pathogenesis	2000	4.66	**2009**	2011	
Necrotizing enterocoliti	2000	6.62	**2011**	2019	
Management	2000	5.41	**2012**	2021	
Survival	2000	5.25	**2013**	2015	
Perfusion	2000	6.51	**2014**	2018	
Intestinal microcirculation	2000	5.35	**2015**	2021	
Cell	2000	5.75	**2017**	2021	
Inflammation	2000	5.2	**2018**	2021	
Gut microbiota	2000	5.74	**2019**	2021	

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Figure 7 Top 25 keywords with the strongest citation bursts. Keywords bursts identify as indicators of emerging trends in the intestinal microcirculatory field to a published article. In the burst detection, "begin" represented the year the reference began to have a citation burst, and "end" represented the year ended the citation burst. The red line is the time of duration and the "strength" is the intensity of the citation burst.

reported the results of a roundtable organized in Amsterdam, reaching a formal consensus on the acquisition and analysis of microcirculatory images. These references provided a solid theoretical foundation for future research.

Based on the analysis of keywords, we sought to identify research interests and focus related to the intestinal microcirculation. Keywords revealed by the co-occurrence map were classified into two categories: Pathophysiology-related research and clinical disease-related research, which is also consistent with the clustering of the cocitation references. The keywords "expression", "blood flow", "microvascular endothelial cell" and "nitric oxide" were associated with the pathophysiology of the intestinal microcirculation. Endothelial cell activation, hemorheological alterations, and altered vasoreactivity were just a few examples of functional and structural modifications[18-20]. Additionally, pathological situations significantly disrupted the nitric oxide (NO) system, which is essential to the autoregulatory control of microcirculatory patency and could result in pathological flow shunting. These conspicuous keywords indicated that further research on microcirculatory mechanisms is needed.

Previous research has revealed that intestinal microcirculatory dysfunction can occur early in patients with shock and sepsis[21]. Necrotizing enterocolitis and inflammatory bowel disease are included as examples resulting from the pathologic changes in the intestinal microcirculation[22]. Several studies have shown that microvascular remodeling and angiogenesis, vasodilatation microvascular dysfunction, and infiltration of immune cells play a role in the pathogenesis of inflammatory bowel disease and necrotizing enterocolitis[23-25]. Additionally, the imbalance among vascular mediators such as NO, catecholamines, and endothelin regulates neonatal intestinal vascular resistance and may influence the pathophysiology of these gut diseases[26,27]. Thus, the intestinal microcirculation as a new therapeutic target offers possibilities for treating these diseases.

Largely ignored throughout history, the intestinal microcirculation has recently been identified as the center of various pathophysiological processes. The determinants of oxygen delivery, tissue oxygen tension, blood flow regulation, and mitochondrial well-being have yet to be fully understood. The origin of intestinal microcirculatory failure in necrotizing enterocolitis and inflammatory bowel disease that is not responsive to therapy is represented by the dysfunction of the integrated intestinal microcirculation rather than systemic hemodynamic variables. Therefore, a new area of outcomes and the potential for discovering novel therapeutic targets has been made possible by introducing improved tools into clinical practice that permit the examination of integrated intestinal microcirculation. Small-molecule drugs (melatonin[28], L-citrulline[29], heparin[30], and potential vasoactive Chinese traditional medicines such as Weiqi decoction[31]), as well as novel therapeutic approaches such as remote ischemic conditioning[32] are recommended. Furthermore, research that determines whether these medicines are effective at enhancing the outcome of patients by ameliorating the intestinal microcircu-

lation should be investigated in the future.

However, our study also has certain limitations. First, this analysis was restricted to English papers in the WOSCC database which may contain fewer established articles than other databases, future research may consider embedding expanded literature databases, such as Scopus. Second, non-English literature was not included in the database or analysis, possibly resulting in linguistic source bias. Finally, bibliometric data that change over time might lead to a different conclusion. In an updated analysis, it will be necessary to follow the most recent primary studies and non-English investigations dynamically.

CONCLUSION

The intestinal microcirculation has important academic value and clinical application prospects in health and diseases. We illustrated the global developing trends, influential articles, thematic keywords, and research frontiers from 2000 to 2021 in this field. In coauthorship analyses, the patterns of scientific cooperation were found across countries/regions, institutions, and journals. Moreover, the current state and potential future directions were detected by the reference cocitation analysis, burst references, and keyword identification. We now have a deeper grasp of the pathophysiologic mechanisms underlying the intestinal microcirculation, and optimal diagnosis, prognosis assessment, and clinical therapies are the features and trends of the field. Multidisciplinary collaborations will be critical to advancing intestinal microcirculatory research.

ARTICLE HIGHLIGHTS

Research background

The intestinal microcirculation plays an important role in food absorption and metabolic substance exchange. And it is beneficial to comprehensively describe the progress of intestinal microcirculation research and provide information that may guide future research efforts.

Research motivation

Few attempts have been made to systematically assess scientific findings and current networks in the field of intestinal microcirculation. It is difficult to identify potential research hotspots or emerging research frontiers.

Research objectives

To investigate the research status, development trend, and frontier dynamics of intestinal microcirculation in the past 22 years (2000-2021).

Research methods

Based on the core literature published in the Web of Science database from 2000-2021, VOSviewer and CiteSpace 6.1.R2 were used to analyze and visualize the overall characteristics, source countries, institutions, journals, and citation frequencies of intestinal microcirculatory research.

Research results

A total of 1364 publications were included in the bibliometric analysis, showing an upward trend from 2000 to 2021. The United States and Dalhousie University ranked first among all countries and institutions. Most of the publications were released in Shock, and the most cited journal was Nature Reviews Microbiology Clinical. The topical hotspots and frontiers of intestinal microcirculation focused on the pathological processes of functional impairment on intestinal micro-vessels, diverse intestinal illnesses, and clinical treatment.

Research conclusions

Our study reveals research trends in the field of intestinal microcirculation and offers serviceable guidance to researchers by providing the prolific areas for intestinal disease research to date.

Research perspectives

Our analysis systematically assesses the scientific findings and current networks in this study of intestinal microcirculation from a worldwide perspective. Optimization of diagnosis, prognostic assessment, and clinical treatment are features and trends in this field. Multidisciplinary collaboration is essential to facilitate intestinal microcirculation research.

FOOTNOTES

Author contributions: Liu MM designed the scientometric analysis strategy; Fu SJ, Xu MT, Wang B, Li BW, Ling H, Wang Q, Liu XT, Zhang XY, and Li AL downloaded and analyzed the data; Fu SJ and Liu MM wrote the manuscript; Liu MM made critical revisions to the article for important intellectual content; All authors discussed the results and approved the final version of the manuscript.

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LETTER TO THE EDITOR

Thiopurines are an independent risk factor for active tuberculosis in inflammatory bowel disease patients

Flora Maria Lorenzo Fortes, Raquel Rocha, Genoile Oliveira Santana

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Abstract

The use of thiopurines is an independent risk factor for active tuberculosis in patients with inflammatory bowel disease.

Key Words: Tuberculosis; Inflammatory bowel disease; Thiopurines; Therapy; Risk

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Core Tip: Inflammatory bowel disease (IBD) patients recommended for anti-tumor necrosis factor (anti-TNF) therapy need to be tested for latent tuberculosis (TB) prior to treatment. Azathioprine monotherapy is also an independent risk factor for active TB in patients with IBD. However, the recommendations of the Brazilian Public Health Guideline for Tuberculosis Prevention do not include patients who are receiving immunosuppressive therapy in the risk group for screening for latent TB. We evaluated 301 patients with IBD, and the use of azathioprine treatment increased the risk by 6.87-fold compared to patients without this treatment. The use of anti-TNF therapy had a 10.34-fold increased risk of TB, and the combination of both increased the risk by 17.81-fold.

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TO THE EDITOR

It is known that immunosuppression increases the risk of tuberculosis, especially in countries with a high frequency of active tuberculosis. We read with interest the article published by Fortes *et al*[1], who performed a retrospective cohort study among Inflammatory bowel disease (IBD) patients at a reference center in Brazil, which is a country with a moderate incidence of TB. A total of 301 IBD patients were evaluated; 61.8% had ulcerative colitis, and 38.2% had Crohn's disease. Twenty-seven (9.0%) patients received anti-tumor necrosis factor (anti-TNFα) as a monotherapy, 31 (10.3%) patients received anti-TNF α associated with azathioprine, 3 (1.0%) patients received anti-TNFα treatment associated with methotrexate, and 70 (23.3%) patients received only azathioprine. The use of azathioprine treatment increased the risk by 6.87-fold in comparison to patients without this treatment. The use of anti-TNF therapy showed a 10.34-fold increased risk of TB in this sample, and the association of both increased the risk to 17.81.

Advances in the treatment of IBD have been adopted worldwide. Some post marketing adverse events have been reported, including active tuberculosis (TB) during anti-TNF therapy. It has already been established that the incidence of active TB in this scenario is associated with the TB burden in the geographic region of the study. Brazil is one of the 20 countries in which TB presents a high incidence along with countries from Africa and Asia[2].

IBD patients with a recommendation for anti-TNF therapy need to test for latent TB before treatment. The TNF alpha blocking mechanism, which is critical in stabilizing granulomas during TB infection, would explain this increase in risk. An unanswered question is whether azathioprine in monotherapy is an independent risk factor for active TB in IBD patients[1,3]. A case report published by van Wijngaarden *et al*[4] already drew attention to the development of pleural tuberculosis in a patient with Crohn's disease while receiving azathioprine as the sole immunosuppressive treatment.

Considering that transplant recipients need substantial immunosuppression and azathioprine is one of the drugs used, studies among transplant recipients receiving immunosuppressive therapy helped guide physicians in the care of IBD patients. A Spanish group evaluated the risk factors for active TB after lung transplantation and concluded that the use of azathioprine was identified as an independent risk factor[5].

The recommendations of the Brazilian Public Health Guideline for Tuberculosis Prevention, reviewed in 2020, did not include patients receiving immunosuppressive therapy in the risk group for screening of latent TB[6]. However, consensus from endemic countries suggests investigation and treatment of latent TB before starting immunosuppressive therapy[7-9].

These findings suggest that in areas with a high burden of TB, the use of thiopurines is an independent risk factor for active TB in IBD patients. This evidence needs to be considered when using this therapy for these patients, especially those from countries with a high TB burden. We suggest giving attention to and treating patients with latent tuberculosis and guiding prevention with possible contacts with active tuberculosis. New studies reporting the risk of active TB among IBD patients receiving immunosuppressive therapy from countries with different incidence rates of TB are needed.

FOOTNOTES

Author contributions: Fortes FML designed the study and performed the data analysis; Rocha R reviewed the manuscript and provided technical and material support; and Santana GO contributed to the study design, manuscript revision, supervision of the study, had full access to all of the data in the study and was responsible for the integrity of the data.

Conflict-of-interest statement: Genoile O Santana is on the Advisory Board for Janssen; has received speaking fees from Abbvie, Ferring, Janssen, Takeda, Pfizer and UCB Pharma; and has received research grants from Janssen, Lilly, Pfizer, Roche and Takeda. The other authors declare that they have no conflicts of interest.

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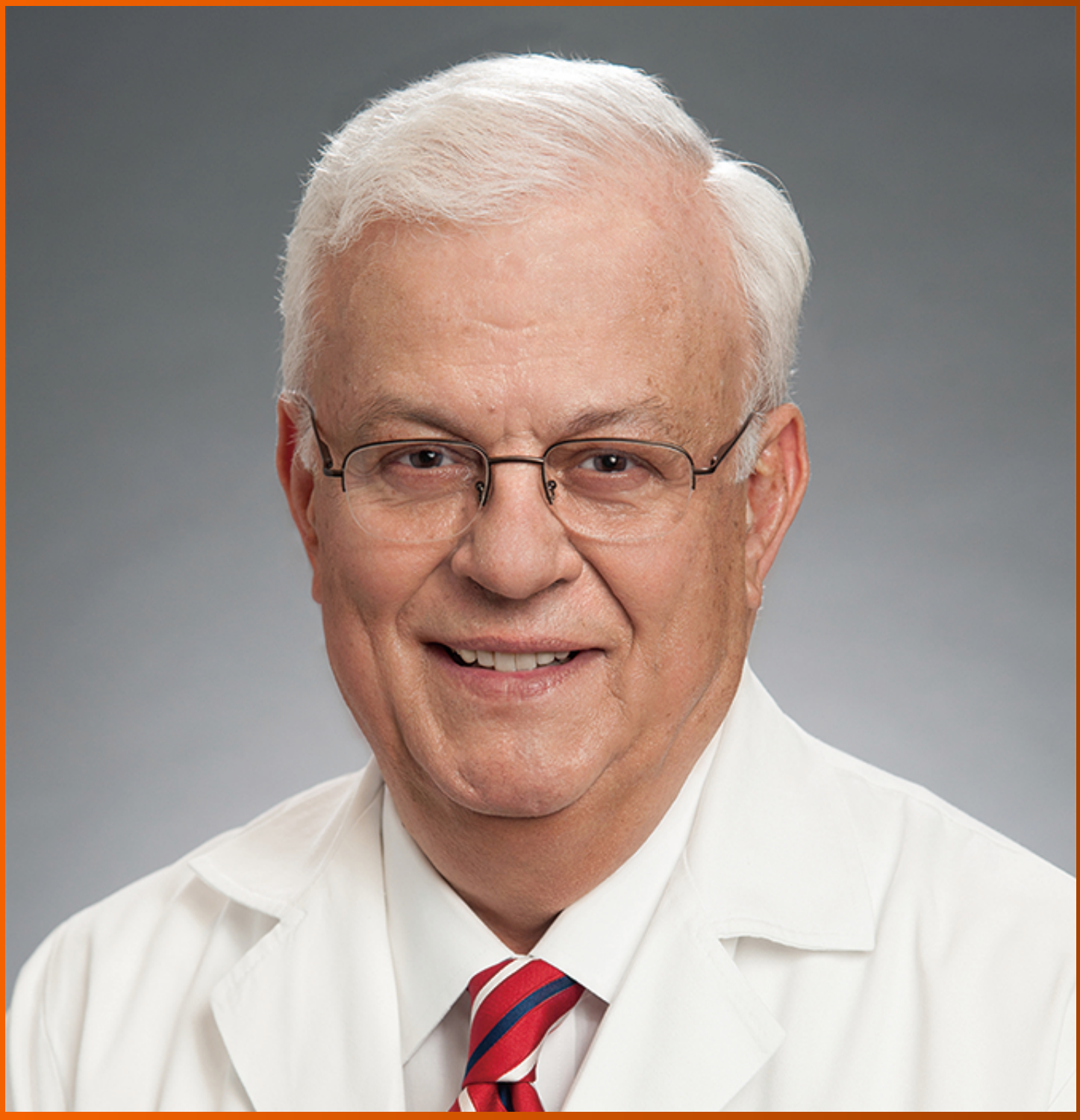


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REVIEW

Precision medicine in inflammatory bowel disease: Individualizing the use of biologics and small molecule therapies

Eric Cheah, James Guoxian Huang

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Abstract

The advent of biologics and small molecules in inflammatory bowel disease (IBD) has marked a significant turning point in the prognosis of IBD, decreasing the rates of corticosteroid dependence, hospitalizations and improving overall quality of life. The introduction of biosimilars has also increased affordability and enhanced access to these otherwise costly targeted therapies. Biologics do not yet represent a complete panacea: A subset of patients do not respond to first-line anti-tumor necrosis factor (TNF)-alpha agents or may subsequently demonstrate a secondary loss of response. Patients who fail to respond to anti-TNF agents typically have a poorer response rate to second-line biologics. It is uncertain which patient would benefit from a different sequencing of biologics or even a combination of biologic agents. The introduction of newer classes of biologics and small molecules may provide alternative therapeutic targets for patients with refractory disease. This review examines the therapeutic ceiling in current treatment strategies of IBD and the potential paradigm shifts in the future.

Key Words: Precision medicine; Therapeutic ceiling; Inflammatory bowel disease; Biologics; Small molecules

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Core Tip: Precision medicine and individualizing patient care has been the holy grail in the management of inflammatory bowel disease (IBD). A one-size-fits-all approach, utilizing the current armamentarium of biologics and small molecules, still yields less than ideal clinical outcomes, with significantly high non-response rates. Multiple challenges remain in breaking this therapeutic ceiling: Achieving an early diagnosis of IBD ideally even in the pre-clinical phase; accurately prognosticating the disease course; and tailoring an appropriately sequenced therapy regime to a patient's disease severity, pharmacokinetic and pharmacodynamic profile.

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INTRODUCTION

The incidence of inflammatory bowel disease (IBD) has seen a rise within Asia Pacific. Overall incidence and prevalence rates in Asia are lower than the West, but are on the rise[1,2]. Treatment of IBD has progressed rapidly over the past several decades. A new era in the treatment of IBD began with the development of the chimeric monoclonal anti-tumor necrosis factor (TNF)-alpha antibody cA2 in the early 1990's. cA2 was subsequently renamed infliximab, and was first licensed by the United States Food and Drug Administration (FDA) in August 1998 for the treatment of Crohn's disease (CD)[3]. Since the introduction of infliximab, there has been an advent of newer biologics and small molecule agents, along with paradigm shifts in the treatment goals of IBD. The agents currently FDA approved for use include the anti-integrin (vedolizumab), anti-interleukin (IL)-12/23 p40 (ustekinumab), anti-IL-23p19 (risankizumab), oral Janus kinase (JAK) inhibitors (tofacitinib, upadacitinib), and sphingosine-1-phosphate (S1P) receptor modulator (ozanimod). As of 2019, mesenchymal stem cell therapy (darvadstrocel) received a regenerative medicine advanced therapy designation for complex perianal fistulas in adult patients with CD. New targets and treatments being explored in Phase II/III trials include anti-integrin (etrolizumab, ontamalimab), IL-23p19 inhibitors (mirikizumab, brazikumab, guselkumab), oral JAK inhibitor (filgotinib), and S1P modulator (etrasimod)[4].

Current treatment goals in IBD aim to more than just achieve clinical remission. Deep remission, the combination of clinical remission and mucosal healing, represents an important therapeutic target that is now increasingly attainable with the timely use of biologics[5]. Expert consensus statements in the STRIDE[1]/STRIDE-II guidelines, with evidence from the CALM study, have helped us define treat-to-target strategies in adults and children utilizing clinical indices, biomarkers, and endoscopic parameters [6-8]. Aspirational targets include transmural healing in CD and histologic healing in ulcerative colitis (UC).

There is an increasing need to develop newer biologics and small molecules targeting novel cytokine pathways, as current therapeutic options are far from perfect in achieving the above-mentioned treatment targets. A meta-analysis of real-world deep remission rates with anti-TNF agents demonstrated that deep remission was only achieved in 48.6% of CD patients and 43.6% of UC patients at 1 year[9]. In a review by Papamichael *et al*[10], the rates of primary non-response and non-remission to anti-TNF agents in IBD were between 10%-40% and 50%-80%, respectively. A further 23%-46% of those initial responders or those who achieve remission have a secondary loss of response over time [11]. The clinical remission rates with second-line biologics are also poorer in patients, who have had a prior loss of response to anti-TNF agents, especially those who had a primary non-response[12-14]. Such data distinctly highlight the therapeutic ceiling in current IBD management: how can we optimize current therapies to go beyond this therapeutic ceiling?

BIOMARKERS IN IBD

Predictive and prognostic biomarkers, pharmacogenomics, and response to therapy

Precision and personalized medicine has long been a discussed topic in the management of IBD. It is an aspirational goal to accurately predict those with a complicated and aggressive clinical course, and to administer timely targeted therapy to the individual's molecular inflammatory profile[15]. Particularly for CD, the emphasis is for appropriately early management within the window of opportunity, before permanent digestive damage is done[16,17]. However, management decisions are still currently made using a one-size-fits-all approach. The conventional strategy is the step-up approach, which will inevitably undertreat patients who are destined to run a more aggressive disease course. With easier

access to biologics and small molecules, a top-down approach may be used, which has been shown to improve clinical outcomes in prognostically severe CD[18,19] but this may otherwise expose patients destined to have mild disease to unnecessary risks and overtreatment. This top-down strategy would also be unaffordable in financially constrained health-care settings particularly in the Asia-Pacific region, and may pose a challenge in health jurisdictions that limit the use of expensive novel therapies.

Furthermore, the heterogeneity and variability of the clinical course of IBD between different individuals would mean a suboptimal approach in a substantial group of patients. There is a general consensus that a tailored approach is required, with a need for accurate biomarkers that enable the right patient to be matched to the right treatment (Table 1).

Stratification of treatment approaches can help identify those of a more complicated course and hence tailoring treatment accordingly. Among prognostic biomarkers at diagnosis predicting a more complex CD phenotype and worse outcomes including stricturing phenotype and need for surgery, identified microbial predictors include circulating antibodies against bacterial antigens such as anti-outer membrane protein C, anti-*Saccharomyces cerevisiae* antibody, perinuclear anti-neutrophil cytoplasmic antibodies, and anti-CBir1 flagellin. However, it is unclear if these represent a cause or effect of severe disease[20-24].

A CD8+ T-cell clonal signature was identified to predict worse outcomes and relapse in IBD patients [25,26]. This genomic biomarker was subsequently validated in independent cohorts of newly diagnosed CD and UC patients in the United Kingdom[27]. There is now a trial in progress in the United Kingdom to assess this whole-blood biomarker to guide treatment for newly diagnosed CD patients[28]. It is currently available in clinical use: PredictSure IBD; PredictImmune, Cambridge, United Kingdom.

Other predictive tools include the use of fecal calprotectin as a predictor of endoscopic disease activity as well as histologic inflammation, and fecal calprotectin values have been shown to be predictive of relapse in asymptomatic patients with IBD[29-33]. In a biomarker discovery trial, the EMBARK study, serum matrix metalloproteinase 9 and serum IL-22 were found to be associated with inflammatory disease activity for patients with UC and CD respectively[34].

Pharmacogenomic testing has also become common place in IBD. A commonly used predictor of risk of adverse drug reactions and pharmacologic response is the utility of thiopurine methyltransferase (TPMT) genotyping and metabolite testing, as well as nudix hydrolase 15 (NUDT15) genotyping. Thiopurine use is associated with adverse effects (AEs) in up to 40% of patients[35]. TPMT genotype testing is cost effective, and heterozygous and homozygous TPMT genotypes correlate with AEs. Dose reduction in the TPMT variants significantly reduce adverse hematologic effects without reducing treatment efficacy[36]. NUDT15 variants, first elucidated in a Korean population, have also been more recently described as associated with thiopurine induced myelosuppression and is more predictive of myelosuppression in East Asians[37-39]. Furthermore, thiopurine metabolite testing can aid in dose optimization and compliance[40] and prevents hepatotoxicity by identifying a subgroup of thiopurine-'shunters' who preferentially produce the hepatotoxic metabolite 6-methylmercaptopurine.

Apart from this, several biomarkers as predictors of non-response to anti-TNF include higher oncostatin M expression[41,42] and low expression of triggering receptor expressed on myeloid cells 1 [43,44]; antibody formation to anti-TNF is associated with the HLA-DQA1*05 genotype[45]. Higher baseline concentrations of serum cytokine IL-22, whose expression is induced by IL-23, is associated with greater likelihood of response to brazikumab[46].

OPTIMIZING AND MAXIMIZING CURRENT BIOLOGIC AGENTS

Therapeutic drug monitoring and drug dosing strategies

Introduction of therapeutic drug monitoring (TDM) has guided our approach in going beyond the therapeutic ceiling. Multiple studies have demonstrated an association with serum drug concentration of biologics, mainly in anti-TNF agents, and outcomes of patients[47-55]. It has assisted us in guiding dose modification (dose escalation or reduction), and informed us of primary or secondary loss of response, thus avoiding persistence of potentially ineffective therapy[56]. The approach of proactive *vs* reactive therapeutic drug monitoring remains a hotly debated topic[57].

Dashboard systems are clinical decision support tools utilizing computer software modelling to predict ideal personalized medication dosing[58,59]. This 'model-based dosing' has long been used by pharmacists and pharmacologists, for example, to dose antibiotics such as aminoglycosides. For anti-TNF dosing, dashboard driven pharmacokinetic (PK) dose optimization considers individual patient covariates including C-reactive protein, albumin, body-weight, sex and also serum drug levels. The PRECISION trial demonstrated that dashboard driven personalized dosing resulted in a significantly higher proportion of patients maintaining clinical remission after 1 year of treatment compared with patients that continued treatment without proactive adjustments: 88% *vs* 64%, respectively[60]. Utilizing the same PK dashboard system during Infliximab induction, Dubinsky *et al*[61] recently showed improved infliximab durability; and at 52 wk, 119/123 patients remained on infliximab in steroid free remission. The OPTIMISE trial is underway to evaluate the safety and efficacy of proactive TDM

Table 1 Selected list of biomarkers in inflammatory bowel disease

Biomarker class	Biomarker	Clinical utility
Prognostic biomarkers	Anti-ompC, ASCA, ANCA, anti-CBir1, flagellin	Prediction of more severe CD phenotype- particularly stricturing and need for surgery
	CD 8+ T cell clonal signature	Prediction of more severe disease course and relapse in CD and UC
Surveillance of disease activity	Fecal calprotectin	Predictor of endoscopic disease activity as well as histologic inflammation, and relapse in asymptomatic patients with IBD
	MMP-9	Associated with disease activity in UC
	IL-22	Associated with disease activity in CD
Pharmacogenomics and prediction of safety	TPMT	Risk of thiopurine adverse reaction
	NUDT15	Risk of thiopurine adverse reaction, more common in East Asian/Asian populations
	Thiopurine metabolites (6TG, 6MMP)	Levels associated with adverse drug reaction: myelosuppression, hepatotoxicity. 6TG range also associated with therapy response
Prediction of response to therapy	Oncostatin M	Higher levels predictor of non-response to anti-TNF
	TREM-1	Low levels predictor of non-response to anti-TNF
	HLA-DQA1*05	Expression associated with risk of antibody formation to anti-TNF
	IL-22	Higher level associated with response to anti-IL23p19 (brazikumab)

ANCA: Anti-neutrophil cytoplasmic antibody; ASCA: Anti-Saccharomyces cerevisiae antibody; CD: Crohn's disease; IBD: Inflammatory bowel disease; IL: Interleukin; MMP: Matrix metalloproteinase; NUDT15: Nudix hydrolase 15; ompC: Outer membrane protein C; TG: Thioguanine; TNF: Tumor necrosis factor; TPMT: Thiopurine methyltransferase; TREM: Triggering receptor expressed on myeloid cells; UC: Ulcerative colitis.

combined PK dashboard-driven infliximab dosing compared with standard of care dosing in patients with CD[62].

"Supratherapeutic" anti-TNF dosing

Numerous exposure-response relationship studies including post-hoc analyses of randomized controlled trials show a positive correlation between biologic drug concentrations and favorable therapeutic outcomes in IBD and other immune-mediated inflammatory diseases; higher drug concentrations are typically associated with improved therapeutic outcomes[63]. Conversely, lower drug concentrations, with or without anti-drug antibodies, are associated with treatment failure and drug discontinuation[64,65].

Multiple clinical studies have provided various TDM targets, especially with the anti-TNF agents infliximab and adalimumab, at various time points that are associated with outcomes of clinical remission[51,66]. Several observational studies have suggested that higher median infliximab concentrations are associated with superior clinical and biochemical remission rates. Given the wide variation in observed concentrations among responders, one may even wonder if the "therapeutic threshold" is identical for all patients and for the different phases of the treatment (induction *vs* maintenance and active *vs* quiescent disease).

Yarur *et al*[67], found that that levels of infliximab ≥ 10 mcg/mL were best associated with fistula healing, though surprisingly, a small number of patients required levels of ≥ 20 mcg/mL to achieve fistula healing. Feng *et al*[68] demonstrated that on incremental gains analysis, mucosal healing rates gradually increased as infliximab levels went up and reached a brief plateau (> 85%) when the infliximab trough level was 10 μg/mL. However, there was still a small proportion that seemingly benefited from an anti-TNF levels > 12 mcg/mL to achieve mucosal healing. Ungar *et al*[69] similarly demonstrated in a retrospective study a significant association between serum levels of anti-TNF agents and level of mucosal healing. They went on to propose that serum levels of 6-10 μg/mL for infliximab and 8-12 μg/mL for adalimumab are required to achieve mucosal healing in 80%-90% of patients with IBD.

Several other studies demonstrated that higher trough levels of infliximab and adalimumab are associated with those achieving mucosal healing in CD[68-79]. In a substudy of the TAILORIX trial, Bossuyt *et al*[71] found that infliximab trough level of 7.8 μg/mL at the end of induction (week 14) was associated with both radiologic response and remission. Continuously high infliximab exposure (infliximab > 5 μg/mL at all time points) was associated with radiologic response.

Low infliximab trough concentrations and the presence of antibodies to infliximab are associated with worse outcomes. Trough concentrations of > 3 μg/mL during maintenance is associated with sustained clinical outcomes[54,70,80,81]. Vande Casteele *et al*[82] concluded that an appropriate infliximab therapeutic window is between 3 and 7 μg/mL for IBD responders during maintenance therapy based on previous studies, and prospectively validated it in a randomized controlled trial (TAXIT).

The optimal "therapeutic window" for biologics remains to be elucidated, and the upper limit is unclear. While the abovementioned studies observed an association between certain trough concentration ranges and a corresponding degree of disease remission, the 'ideal' trough concentration to induce remission may vary between individuals. This may be due to a variety of factors such as an individual's disease burden and therefore mucosal TNF burden, early *vs* advanced disease, and an individual's unique pharmacodynamic makeup. There may be a subset of patients who might benefit from dose escalation to 'supratherapeutic' trough concentrations and reassuringly, there is little evidence to indicate greater toxicity with higher infliximab levels.

Sequencing of biologics

While it remains a conventional strategy to offer an anti-TNF agent as the first-line biologic, it has been well established from network meta-analyses that anti-TNF non-responders do not have an optimal response after switching to second-line biologics[12-14]. It thereby raises the question whether certain individuals would benefit from receiving these traditionally second-line biologics as first-line therapy options, and highlights the importance of optimal biologic sequencing.

There are limited head to head trials between biologic agents: In the VARSITY trial (adalimumab *vs* vedolizumab) for moderate to severe UC, vedolizumab demonstrated superiority in clinical and endoscopic remission but not corticosteroid free clinical remission[83]. In the SEAVUE study (adalimumab *vs* ustekinumab) for moderate to severe Crohn's, ustekinumab failed to demonstrate superiority over adalimumab[84].

In the Galaxi-1 study involving participants with moderately to severely active CD, guselkumab was compared to placebo and ustekinumab. It was a phase 2, dose-ranging study[85], not powered to evaluate potential differences in efficacy and safety between guselkumab and ustekinumab.

From the HIBISCUS and GARDENIA trials[86,87] Etrolizumab *vs* adalimumab or infliximab in moderate to severe UC- failed to demonstrate superiority.

While offering novel alternate pathway biologics as first-line therapy may gain traction in the near future, this approach is often limited by government access in many jurisdictions. Access to newer therapies for adult patients is already limited by licensing authorities, but the pathways remain even more restricted for pediatric patients. Access to biologics in pediatric patients is often on compassionate grounds, due to a significant lag in clinical trials and therefore delaying official approval from medical licensing authorities such as the European Medicines Agency and United States FDA. There is a need for this cohort of patients to have better access to new/emerging therapies through more advanced pharmacogenomic, pharmacokinetic and pharmacodynamic modelling. This is to allow earlier initiation of trials or better ways to "extrapolate" adult data for presentation to licensing authorities to allow for use in children.

Dual biologics and combinations of newer advanced therapies

Combinations of biologic agents and recently combinations of biologics with newer small molecule agents have been attempted to go beyond our current therapeutic ceiling. This concept is not new, however, and was first attempted by Sands *et al*[88] as a randomized controlled trial comparing the safety and tolerability of patients on infliximab not in remission and adding natalizumab *vs* a placebo arm. Although the main trial ran for 10 wk and was not powered to assess for differences in efficacy between the groups, there was a higher proportion of patients achieving a clinical response at each time point and this proportion continues to increase over time in the combination biologic group, compared to response rates for the monotherapy arm which remained unchanged.

Since then, there has been much in the literature of dual biologics or with newer small molecule therapies, but as case reports or case series and observational cohort studies (Table 2). The data is largely heterogenous but reassuringly has demonstrated acceptable safety for patients with refractory IBD with no new concerning signals[89,90].

In the pipeline, there are several randomized controlled trials evaluating the efficacy of combination biologics. The EXPLORER trial (ClinicalTrials.gov Identifier: NCT02764762) in high-risk CD patients involved triple combination therapy with vedolizumab, adalimumab and methotrexate which has completed but not yet reported.

The phase 2a VEGA study evaluated the safety and efficacy of combination induction therapy with guselkumab plus golimumab (GOL) *vs* monotherapy with guselkumab or GOL in adults with moderately to severely active UC through to week 12, most recently presented at the European Crohn's and Colitis Organization 2022 congress[91,92]. A greater proportion of patients who received combination therapy achieved clinical response as judged by Mayo score at week 12 (83.1%) *vs* guselkumab (74.6%) or GOL (61.1%). Similarly, the proportion of patients who achieved clinical remission in the combination group (36.6%) was greater than that in the monotherapy group (21.1% and 22.2%, respectively). The DUET UC (ClinicalTrials.gov Identifier: NCT05242484) is a phase 2b study of

Table 2 Publications of dual biologics

Ref.	Type	Number of participants/IBD type	Biologic combinations	Therapy duration or follow up (mo)	Outcomes
Buer *et al* [97], 2018	Case series, prospectively followed	Adult: 10 (4 CD, 6 UC)	Anti-TNF, adding on vedolizumab. Combination was intended as a bridging therapy	12-20	Clinical: HBI, PMS, 100 % CRem, 50% endoscopic remission. No serious AE (3 minor infections)
Olbjørn *et al*[98], 2020	Case series	Pediatric: 13 (9 CD, 4 UC)	Anti-TNF + vedolizumab (8), anti-TNF + ustekinumab (5), (for anti-TNF side effects)	N/A	3/8 (37.5%) Clinical and biochemical remission
Kwapisz *et al*[99], 2021	Case series	Adult: 15 (14 CD, 1 UC)	8 vedolizumab + anti-TNF, 2 ustekinumab + anti-TNF, 5 vedolizumab + ustekinumab	24	73% CRes, 44% ERes, 27% SE, 20% surgery
Yang *et al* [100], 2020	Retrospective cohort	Adult: 22 (CD)	24 combinations: 13 vedolizumab + anti-TNF, 8 vedolizumab + ustekinumab, 3 ustekinumab + anti-TNF	1	Endoscopic, PRO2 response/remission, CRP, 50% CRes, 36% SF CRem, 43% ERes, 4% SE (1 SLE-1 cancer), 33% surgery
Glassner *et al*[101], 2020	Retrospective cohort	50	53 combinations: 25 vedolizumab + ustekinumab, multiple other combinations	5.5-13	50% CRem, 34% ERem, 16% SE, 12% surgery
Privitera *et al*[102], 2020	Case series, indication active IBD and active EIM	Adult: 16 (11 CD, 4 UC)	Variety of combinations. Most frequent: 3 vedolizumab + adalimumab, 3 vedolizumab + ustekinumab	0.5	At 6 mo: Response IBD/EIM: 42.8%, 11%; Remission IBD/EIM: 14.2%, 55.5%, AE: 3/16 (18.8%)
Dolinger *et al*[103], 2021	Case series	Pediatric: 16: (CD 7, UC 8, IBD-U 1)	Vedolizumab + ustekinumab, vedolizumab + tofacitinib, ustekinumab + tofacitinib	6	SF remission at 6 mo 12/16 (75%)
Goessens *et al*[104], 2021	Retrospective cohort, hetero-genous, active IBD and/or EIM	Adult: 98 (CD 58, UC 40)	Anti-TNF + vedolizumab, anti-TNF + anti-IL, anti-IL + vedolizumab, tofacitinib + anti-TNF, tofacitinib + vedolizumab, anti-IL + anti-IL, others	5-16	PGA: Complete or partial improvement was observed in 21/80 (26%) and 35/80 (44%); Mean clinical disease activity for IBD: Significantly higher prior to combination than during combination (2.2 +/- 0.7 *vs* 1.2 +/- 1.1; $P < 0.0001$). Simple clinical activity scores (quiescent scores 0, mild scores 1, moderate scores 2 and severe scores 3

AE: Adverse events; CD: Crohn's disease; CRes: Clinical response; CRem: Clinical remission; EIM: Extraintestinal manifestations; ERem: Endoscopic remission; ERes: Endoscopic response; HBI: Harvey Bradshaw index; IBD: Inflammatory bowel disease; IL: Interleukin; N/A: Not applicable; PGA: Physician global assessment; PMS: Partial Mayo score; PRO2: Patient reported outcome scores; SE: Serious infection; SF: Steroid free; TNF: Tumor necrosis factor; UC: Ulcerative colitis.

combination therapy with guselkumab and GOL (JNJ-78934804) in participants with moderately to severely active UC that is planned; the DUET CD (ClinicalTrials.gov Identifier: NCT05242471) is a phase 2b study of the same biologic combination in individuals with moderately to severely active CD, and is currently recruiting trial participants.

The other considerations to overcome current therapeutic plateaus with biologic agents include added adjunctive therapies such as vitamin D, curcumin, microbiome alteration *via* dietary modification, exclusive enteral nutrition and probiotics[93].

CONCLUSION

Ongoing efforts in adding to and optimizing IBD treatments must be commended. However, remission rates are still far from optimal with current treatment approaches. The urgent need to develop new therapeutics also brings us to the challenge that we must meet: Improving the design and delivery of clinical trials, allowing generalizability, be of clinical equipoise and to factor in biomarker discovery. Advances in basic science, translational and clinical aspects of drug development is essential to achieve breakthroughs in IBD therapeutics that meet the needs of patients, physicians and health regulators[94-96]. In conclusion, in addition to the strategies as aforementioned, to go beyond our current therapeutic

ceiling requires not only early diagnosis or early stratification but early treatment. This entails incorporating a multiomics approach to better personalize treatment, sequence or combine our therapies, and incorporate the ever-advancing artificial intelligence technology, rather than a one-size-fits all approach[95]. These goals remain attainable and we continue to have a sense of optimism.

FOOTNOTES

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MINIREVIEWS

Systemic treatment for unresectable hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is most commonly found in the context of liver cirrhosis and, in rare cases, in a healthy liver. Its prevalence has risen in recent years, particularly in Western nations, due to the increasing frequency of non-alcoholic fatty liver disease. Advanced HCC has a poor prognosis. For many years, the only proven therapy for unresectable HCC (uHCC) was sorafenib, a tyrosine kinase inhibitor. Recently, the synergistic effect of an immune checkpoint inhibitor, atezolizumab, and bevacizumab outperformed sorafenib alone in terms of survival, making it the recommended first-line therapy. Other multikinase inhibitors, lenvatinib and regorafenib, were also recommended as first and second-line drugs, respectively. Intermediate-stage HCC patients with retained liver function, particularly uHCC without extrahepatic metastasis, may benefit from trans-arterial chemoembolization. The current problem in uHCC is selecting a patient for the best treatment while considering the preexisting liver condition and liver function. Indeed, all study patients had a Child-Pugh class A, and the best therapy for other individuals is unknown. Additionally, in the absence of a medical contraindication, atezolizumab could be combined with bevacizumab for uHCC systemic therapy. Several studies are now underway to evaluate immune checkpoint inhibitors in combination with anti-angiogenic drugs, and the first findings are encouraging. The paradigm of uHCC therapy is changing dramatically, and many obstacles remain for optimum patient management in the near future. The purpose of this commentary review was to give an insight into current systemic treatment options for patients with uHCC who are not candidates for surgery to cure the disease.

Key Words: Hepatocellular carcinoma; Unresectable hepatocellular carcinoma; Non-alcoholic fatty liver disease; Tyrosine kinase inhibitor; Sorafenib; Lenvatinib; Immune checkpoint inhibitor; Atezolizumab; Bevacizumab

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Core Tip: Hepatocellular carcinoma (HCC) is a major health problem that is the fourth leading cause of cancer-related mortality worldwide. The 5-year survival rate was nearly 19%, but only 2% in metastatic HCC. The first oral multikinase inhibitor for the systemic treatment of advanced or unresectable HCC (uHCC) was sorafenib. However, when compared to sorafenib, the combination of atezolizumab and bevacizumab increased survival rates and was authorized as first-line treatment for uHCC. Regorafenib and cabozantinib are suggested for use as second-line drugs in the event that the disease progresses. Transarterial chemoembolization for palliative care or downstaging is also suggested. This review focused on systemic therapy for uHCC patients who are not appropriate for liver-directed therapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common kind of primary liver cancer and a serious public health concern globally. With 905677 new cases and 830180 fatalities in 2020, liver cancer is the sixth most common malignancy and the third major cause of cancer mortality. The age-adjusted global incidence is 9.5 deaths per 100000 and the mortality rate is 8.7 per 100000 people[1]. HCC typically develops in the presence of cirrhosis, less frequently in chronic liver disease that is not cirrhotic, and very rarely in a healthy liver. The risk factors for HCC are well-known and vary by geographic location. In Asia and Africa, viral hepatitis is the main cause of HCC, but in North America and Western Europe, fatty liver disease and obesity are the main causes. The length of time that these risk factors have been detected in human populations is strongly correlated with the global increase in HCC incidence[2-4]. Depending on the tumor stage and liver function, the prognosis and treatment plan for HCC in cirrhotic livers are determined (Child–Pugh score). With a 5-year relative survival rate of 18.4%, overall survival (OS) is poor. Patients with localized, regional, and metastasis have 5-year survival rates of 33%, 10%, and 2%, respectively[5]. HCC may be treatable in its early stages by resection, liver transplantation, or ablation. However, patients are typically identified at intermediate or advanced stages due to a lack of symptoms.

For patients with HCC, the Barcelona Clinic Liver Cancer (BCLC) staging system employs many factors to direct the treatment course of action. The Child-Pugh score is comprised of the Eastern Cooperative Oncology Group (ECOG) performance status (PS), tumor burden (including portal invasion status and hepatic spread), and an estimation of the underlying liver function that should be estimated in addition to the Child-Pugh score using the Model for End-Stage Liver Disease score in cases of decompensated cirrhosis or the alpha-fetoprotein (AFP) concentration and albumin-bilirubin score in cases of compensated liver disease. HCC patients are classified into four stages: Very early (BCLC 0), early (BCLC A), intermediate (BCLC B), unresectable (BCLC C), and end-stages (BCLC D). Nearly 40% of HCC patients have an early diagnosis, making them eligible for curative procedures such as local radiofrequency ablation or surgery (liver transplantation or hepatic resection). A systemic, chemoembolization, or radioembolization treatment is necessary for more than half of them, which is in the intermediate stage and is unresectable (BCLC B and C)[6].

Unfortunately, systemic therapy is the only treatment available for individuals with HCC because more than 50% of cases are discovered at an advanced stage. In addition, recurrences occur in around 70% of individuals who have had their main tumor surgically removed[7]. When liver-directed medicines are not a possibility or the patient has experienced a significant recurrence, systemic therapy is chosen. Systemic treatments, including tyrosine kinase inhibitors (TKIs), monoclonal antibodies, and immune checkpoint inhibitors, have been made possible by recent technological advancements. The FDA has officially authorized sorafenib and lenvatinib as first-line therapies for advanced HCC. Second-line treatments for individuals who advanced or did not tolerate sorafenib include cabozantinib, regorafenib, ramucirumab, nivolumab, and pembrolizumab. The United States FDA recently approved the use of neurotrophic tyrosine receptor kinase (NTRK) inhibitors, larotrectinib or entrectinib, in HCC patients with NTRK fusion-positive solid tumors. Although cytokine treatment [interferon alpha-2b, interleukin (IL)-12] produced disappointing results, nivolumab and pembrolizumab have shown promising outcomes in phase II studies in terms of progression-free survival (PFS)[8-11]. Nivolumab in first-line treatment and pembrolizumab in second-line therapy phase III trials' primary endpoints, however, were not achieved[12,13]. The preliminary findings of a phase III study showed that atezol-

izumab with bevacizumab improved OS and PFS when compared to sorafenib as first-line therapy for HCC[14].

The development of effective HCC therapeutics is complicated by tumor heterogeneity caused by multiple risk factors. Greater understanding of the heterogenic tumorigenic pathways should provide information on tumor biomarkers, genomes, and other tumor characteristics that predict response to targeted treatment in HCC. In this review, we aimed to address the carcinogenesis of HCC, clinical trials that assessed medications targeting these tumorigenic pathways, and future directions of targeted treatment in advanced HCC.

PATHOGENESIS

Several pathways have been associated with the development of HCC. These pathways have served as the primary targets for systemic therapy development.

Mitogen-activated protein kinase pathway

In order to activate or deactivate their target, the mitogen-activated protein kinases (MAPK) phosphorylate either their own dual serine and threonine residues or those present on their substrates. As a common downstream pathway for numerous tyrosine kinase receptors, the MAPK pathway is a biological signaling system that controls crucial physiological processes such as cell proliferation, cell differentiation, stress responses, apoptosis, and immune defense. When external growth stimuli bind to these tyrosine kinase receptors, the MAPK cascade is initiated. An MAP3K stimulates an MAP2K, which in turn activates an MAPK, in an MAPK module. MAPK protein phosphatases, which dephosphorylate both phosphothreonine and phosphotyrosine residues on MAPKs, can inhibit MAPK phosphorylation processes. The most prevalent tyrosine kinase receptors are the insulin-like growth factor receptor, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and epidermal growth factor receptor (EGFR). Growth factors that bind to these receptors cause a phosphorylation cascade, which in turn activates the adaptor molecular complex (growth factor receptor-bound protein 2-Src homology and collagen-son of sevenless). The proto-oncogenes rat sarcoma virus (Ras), rapidly accelerated fibrosarcoma (Raf), and guanosine triphosphatase are subsequently activated by this complex, leading to the activation of mitogen-activated protein kinase (MEK) 1/2 and extracellular signal-regulated kinase (ERK) 1/2 downstream. Eventually, this pathway results in the upregulation of gene transcription, which encourages cell proliferation, through the transcriptional activators c-Jun and c-Fos[15,16]. It has also been demonstrated that ERK phosphorylates proteins are involved in cell proliferation, apoptosis resistance, and angiogenesis in HCC[17,18]. Ras mutations were discovered to occur often in HCC, according to several studies, and overexpression of this pathway, particularly in high-grade tumors, has been reported in HCC[19,20]. Sorafenib, which inhibits the Raf serine/threonine kinase as well as several other receptors, was developed on the basis of this pathway[21,22].

Phosphoinositide 3-kinase–Ak strain transforming–mammalian target of rapamycin pathway

Cell development and regulation also depend on the phosphoinositide 3-kinase (PI3K)-Ak strain transforming (AKT)-mammalian target of rapamycin (mTOR) signaling pathway. Growth factors including epidermal growth factor and IL-2 bind to their associated tyrosine kinase receptors to activate PI3K. Phosphoinositol triphosphate, a lipid second messenger produced by PI3K, activates the serine/threonine kinase AKT (protein kinase B). After that, AKT phosphorylates a number of proteins, including the proapoptotic protein Bcl-2 opponent of cell death, to activate the mTOR family of proteins (BAD). To promote cell cycle progression and unrestricted growth and proliferation, mTOR regulates the phosphorylation of the translational repressor proteins ribosomal protein S6 kinase beta-1 (P70S6 kinase) and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1 or 4E-BP-1)[23-25]. Studies have shown that cancers with abnormal expression of the pathway including phosphatase and tensin homolog (PTEN), AKT, and PS6 are more severe and have a poorer prognosis overall than tissues with normal expression[26,27]. In approximately half of all HCC patients, expression of the tumor suppressor gene product PTEN, which generally suppresses PI3K activity, is significantly decreased. Due to the gene deletion process, PTEN is inactivated, which results in uncontrolled PI3K activity and downstream phosphorylation of proteins that inhibit apoptosis and encourage tumor growth[28,29].

Wingless and Int-1/β-catenin pathway

The Wingless and Int-1 (WNT)/β-catenin signaling system regulates embryonic development, cellular proliferation, and differentiation. It is a highly conserved and carefully regulated molecular process. Notably, growing data suggests that abnormal WNT/β-catenin signaling increases the formation and progression of HCC. The two distinct WNT signaling pathways, known as non-canonical and canonical, with the latter including β-catenin activation. Comprehensive genomic analyses have revealed that β-catenin-encoding CTNNB1 and AXIN1 gain-of-function mutations are present in about 35% of human HCC samples. Human HCCs with activated WNT/β-catenin pathways display unique gene expression

patterns and malignant properties. Through its downstream effectors, activated WNT/β-catenin interacts with a variety of signaling pathways to encourage the development of HCC. As a result, medications that target WNT/β-catenin have being looked into as possible HCC treatments[30,31].

SYSTEMIC TREATMENT FOR UNRESECTABLE HCC

First-line systemic therapy

Single drug multikinase inhibitor: Sorafenib-A multikinase inhibitor (MTKI), sorafenib blocks tyrosine kinases and pathways essential for angiogenesis and cell proliferation. VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, KIT, RET, RAS/RAF/MAPK, FLT-3, and Janus kinase/signal transducer and activator of transcription protein are among the receptors that it inhibits (STAT)[32]. The multicenter phase III European Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) research and the Asia-Pacific trial on OS benefit compared with placebo both supported the efficacy of first-line sorafenib for uHCC patients with Child-Pugh class A cirrhosis. The inclusion criteria for both studies were the same: Advanced HCC with detectable illness, mostly Child-Pugh class A cirrhosis, no prior systemic therapy, adequate hematological, renal, and hepatic function, and a life expectancy of at least 12 wk. Six hundred and two untreated uHCC patients from Europe, North America, South America, and Australia were included in the SHARP study. Two groups of patients were randomly treated with either sorafenib 400 mg (n = 299) twice daily or a placebo (n = 303). Treatment with sorafenib was continued until the illness became worse, the toxicity was too much, or someone passed away. Patients receiving sorafenib experienced response rates of just 2%, compared with only 1% in the placebo group. Clinical outcomes were achieved in 43% and 32% of patients in the sorafenib and placebo groups, respectively (P = 0.002). The project was halted after a second planned interim analysis showed that the median OS with sorafenib was significantly longer at 10.7 mo compared to 7.9 mo with placebo (P < 0.01). With sorafenib, 1-year survival rates were 44%, while with placebo, they were 33% (P < 0.01). The most common treatment-related side effects in people taking sorafenib were diarrhea, weight loss, hand-foot syndrome (HFS), and hypophosphatemia[33] (Table 1).

The Asia-Pacific study, which ran alongside the SHARP trial, was designed to look at the safety and activity of sorafenib in uHCC patients. They randomly assigned 150 of the 226 participants who were drawn from 23 locations in China, South Korea, and Taiwan to treat with sorafenib 400 mg twice daily or a placebo (n = 76). Similar to the SHARP study, sorafenib had a low response rate (3.3% *vs* 1.3% for placebo). Sorafenib had a disease control rate of 35.3% whereas a placebo had a rate of 15.8% (P < 0.001). The median OS periods for sorafenib and the placebo were 6.5 mo and 4.2 mo, respectively (P = 0.014). Despite the fact that both studies utilized the identical eligibility standards and treatment strategy, the Asia-Pacific trial's patients had more extrahepatic disease, more hepatic lesions, a worse PS, more advanced disease, and a higher rate of AFP elevation. Because of this, their sorafenib-related survival in the Asia-Pacific study (6.5 mo) was lower than in the SHARP trial (10.7 mo). In the subgroup analysis, sorafenib showed higher efficacy in patients without extrahepatic spread [hazard ratio (HR): 0.55 *vs* 0.84], with hepatitis C-associated illness (HR: 0.47 *vs* 0.81), and with a low neutrophil-to-lymphocyte ratio (HR: 0.59 *vs* 0.84)[34] (Table 2).

In a subset analysis of the SHARP trial, Bruix *et al*[35] looked at how the etiology of the illness, the tumor load, PS, the tumor stage, and past treatments affected survival, disease control, time to progression (TTP), and safety. Compared to placebo, sorafenib treatment significantly enhanced survival and disease control regardless of the etiology, tumor load, PS, tumor stage, or previous therapy. Except for individuals who were hepatitis B virus (HBV)-positive, sorafenib also reliably reduced the median time to progression (MTP). The relationship between the pre-study liver state and the effects of sorafenib on liver function were investigated in a second subgroup analysis of the SHARP trial data. Individuals with baseline transaminase, AFP, or bilirubin levels elevation showed shorter survival periods than those with normal baseline values, regardless of therapy. Patients with normal or high liver markers experienced the same level of safety. The investigators came up with the conclusion that sorafenib was safe and efficient independent of the liver biomarkers present at baseline and that bilirubin levels, which are used to evaluate hepatic function, were consistent during sorafenib treatment. It should be highlighted that the majority of the trial participants had Child-Pugh class A cirrhosis. Therefore, individuals with more severe cases (Child-Pugh class B and C) should not extrapolate from these findings[36].

Sorafenib was also further studied in two large prospective observational trials, GIDEON and INSIGHT. The GIDEON research, or Global Investigation of Therapeutic DEcisions in HCC and of its Treatment with Sorafenib, examined the safety and tolerability of sorafenib in patients with advanced HCC in actual clinical settings. All treatment choices in this study were made by the patient's attending physician. When therapy first started, out of 2708 people, 72.7% were categorized as Child-Pugh class A, 24.5% as class B, and 2.7% as class C. Sorafenib's starting dosage was 400 mg twice daily. Child-Pugh class A, B, and C patients experienced adverse events (AEs) at rates of 69%, 64%, and 39%, respectively, whereas 9%, 14%, and 3% of patients experienced serious medication responses. The most frequent AEs were HFS (32%, 17%, and 5%), diarrhea (28%, 26%, and 11%), and exhaustion (16%, 14%, and 14%) in

Table 1 Summary of phase 3 trials of sorafenib and lenvatinib for treatment of unresectable hepatocellular carcinoma

Ref.	No. of patients (Child-Pugh A/B)	Treatment or placebo	Median OS in mo	Response rate, %	Control rate, %	Median TP in mo	Dose reduction, % patients	Discontinuation due to AE, % patients
Llovet *et al*[33] (SHARP)	298 (284/14)	Sorafenib (400 mg × 2/d)	10.7	2	43	5.5	26	11
	303 (297/6)	Placebo	7.9	1	32	2.8	7	5
Cheng *et al*[34] (Asia-Pacific)	150 (146/4)	Sorafenib (400 mg × 2/d)	6.5	3.3	35.3	5.5	30.9	19.5
	76 (74/2)	Placebo	4.2	1.3	15.8	2.8	2.7	13.3
Kudo *et al*[47] (REFLECT)	478 (478/0)	Lenvatinib (12 mg/d)	13.6	40.6	73.8	7.4	37	9
	476 (476/0)	Sorafenib (400 mg × 2/d)	12.3	12.4	56.4	3.7	38	7

AE: Adverse event; OS: Overall survival; SHARP: Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol; TP: Time to progression.

Table 2 Summary of the 2 large prospective observational studies of sorafenib for treatment of unresectable hepatocellular carcinoma

Ref.	No. of patients (Child-Pugh A or Child-Pugh B)	Treatment	Median OS in mo	Response rate, %	Control rate, %	Median TP in mo	Dose reduction, % patients	Discontinuation due to AE, % patients
Abou-Alfa *et al*[68]	98	Sorafenib (400 mg × 2/d)	10.7	2	43	5.5	26	11
	38	Sorafenib (400 mg × 2/d)	7.9	1	32	2.8	7	5
Cheng *et al*[34] (Asia-Pacific)	150 (146/4)	Sorafenib (400 mg × 2/d)	6.5	3.3	35.3	5.5	30.9	19.5
	76 (74/2)	Placebo	4.2	1.3	15.8	2.8	2.7	13.3

AE: Adverse event; OS: Overall survival; TP: Time to progression.

patients with Child-Pugh classes A, B, and C scores. Patients in Child-Pugh class A had a longer median OS (13.6 mo) than those in Child-Pugh class B or C (5.2 mo) (2.6 mo). The GIDEON study showed that the most prevalent drug-related side effects, as well as sorafenib tolerance, were comparable between Child-Pugh class A and Child-Pugh class B patients. They discovered that some individuals with Child-Pugh class B cirrhosis might receive sorafenib safely. Because of the variety of individuals with Child-Pugh class B conditions, patients should be carefully selected for sorafenib treatment based on a thorough evaluation of their hepatic condition[37].

The INSIGHT study was a prospective multicenter trial that recruited 788 uHCC patients who were treated with sorafenib. The main objective was to assess sorafenib's safety and efficacy, especially TTP and OS. Sorafenib was generally given at a dosage of 800 mg daily. The prevalence of cirrhosis among the patients was 56.7% Child-Pugh class A, 23.3% Child-Pugh class B, and 3.3% Child-Pugh class C. Patients in Child-Pugh classes A, B, and C experienced an OS of 17.6, 8.1, and 5.6 mo, respectively ($P < 0.01$). Sorafenib-related AEs occurred in 64.9% of patients and were regarded as severe in 9.8%. The most frequent serious adverse medication event (5.2%) was diarrhea. HFS (16.5%), nausea (8.0%), and exhaustion (7.9%) were other notable medication side effects. Both MTP and survival decreased dramatically when Child-Pugh scores increased. Patients having a Child-Pugh score of 7 and those with a score of 8 had comparable MTP and survival rates. Patients with a 9 on the Child-Pugh scale had substantially reduced MTP and survival ($P < 0.01$ and $P = 0.003$, respectively)[38].

McNamara *et al*[39] performed a meta-analysis of sorafenib therapy in patients with Child-Pugh class B cirrhosis, including 30 trials and 8678 participants. Most of the investigations were retrospective or prospective single-institution studies. Child-Pugh class A participants had an objective response rate of 4.6%, whereas Child-Pugh class B participants had an objective response rate of 4.2%. The distribution of Child-Pugh status was 79% Child-Pugh class A and 19% Child-Pugh class B. The assessed median OS for Child-Pugh class A patients was 8.8 mo and 4.6 mo for Child-Pugh class B patients. There was an inverse relationship between OS and Child-Pugh class B hepatic dysfunction. Child-Pugh class B

patients had considerably lower survival, according to four studies that compared patients with Child-Pugh classes A and B using several variables ($P < 0.001$). In 35% of individuals with Child-Pugh class A or B, there was grade 3 or 4 toxicities. The rates of treatment termination without progress and treatment-related mortality were comparable. The study's findings revealed that while sorafenib response, safety, and tolerability were unaffected by the Child-Pugh score, survival was strongly impacted by these factors. No research involving patients with Child-Pugh class B cirrhosis were controlled trials, despite the large number of such studies that have been done, and the information that is now available is insufficient to draw firm conclusions on the use of sorafenib in such individuals. This meta-analysis proved that certain individuals with Child-Pugh class B cirrhosis can get sorafenib safely. Patients with Child-Pugh scores of 7 or less seem to be the safest group to treat with sorafenib, and patients with Child-Pugh scores ≥ 8 should be cautioned. Selected Child-Pugh class B patients may benefit from sorafenib even if Child-Pugh class A patients have much higher survival rates than Child-Pugh class B patients.

Other MTKIs such as brivanib, sunitinib, linifanib, erlotinib, and everolimus were also investigated, but none of them demonstrated superiority over sorafenib[40-44]. Prior to regorafenib's approval for second-line therapy in April 2017 and lenvatinib's approval for first-line therapy in August 2018, sorafenib was the sole FDA-approved therapeutic option for uHCC[45,46].

Lenvatinib-Lenvatinib is a MTKI that targets VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, FGFR-1, FGFR-2, FGFR-3, FGFR-4, KIT, and RET. Lenvatinib is approved as the first-line treatment for uHCC patients. Patients who weigh 60 kg or more should take 12 mg daily, while those who weigh less should take 8 mg. Patients with uHCC who have Child-Pugh class A cirrhosis should not have their doses reduced, and there is no suggested dose for those with Child-Pugh class B or C cirrhosis. Based on the REFLECT trial, a noninferiority phase 3 research, lenvatinib was authorized. In this trial, there were 954 untreated patients with uHCC with Child-Pugh class A cirrhosis from 154 locations in 20 nations throughout the Asia-Pacific, European, and North American continents. Lenvatinib at a daily dose of 12 mg ($n = 478$) (for patients' weight > 60 kg) or 8 mg (for patients weighing 60 kg) was given to patients at random, while sorafenib ($n = 476$) was given at a dose of 400 mg twice daily. OS was the primary aim, and patients were treated up until their radiological state worsened or they developed significant toxicity. Response rate, TTP, and PFS were secondary endpoints. Lenvatinib was comparable to sorafenib in terms of survival, with a median OS of 13.6 mo and 12.3 mo, respectively [HR: 0.92; 95% confidence interval (95%CI): 0.79-1.06]. According to baseline features, these effects were discovered to be constant across all patient groupings. In terms of all secondary objectives, lenvatinib was considerably more successful than sorafenib, according to an independent imaging analysis. Response rates were 40.6% *vs* 12.4% ($P < 0.001$) for lenvatinib *vs* sorafenib; disease control rates were 73.8% *vs* 58.4%; MTP rates were 7.4 mo *vs* 3.7 mo ($P < 0.001$); and median PFS rates were 7.3 mo *vs* 3.6 mo ($P < 0.001$). When compared to sorafenib, lenvatinib was related with more incidences of hypertension associated with treatment (42% *vs* 30%), proteinuria (25% *vs* 11%), and hypothyroidism (16% *vs* 2%), and lower rates of alopecia (3% *vs* 25%), HFS (27% *vs* 52%), and diarrhea (39% *vs* 46%). The reductions of dosage, treatment suspensions, and drugs discontinuations were reported by 40%, 37%, and 9% of lenvatinib-treated patients, respectively, and 32%, 38%, and 7% of patients who were treated with sorafenib, respectively. During the REFLECT trial's follow-up period, 33% of patients who were treated with lenvatinib and 39% of patients who were treated with sorafenib received antineoplastic drugs. The median survival time among patients who did not get further treatment with lenvatinib was 11.5 mo and 9.1 mo with sorafenib. Patients who underwent further therapy following lenvatinib therapy and sorafenib therapy had median survival lengths of 20.8 mo and 17.0 mo, respectively. This was one of the earliest signs that sequential treatment might enhance survival[47].

Finn *et al*[48] investigated the relationships between serum or tissue biomarkers and effectiveness outcomes from the REFLECT study. ELISA was used to assess serum biomarkers [VEGF, angiopoietin-2 (ANG2), FGF19, FGF21, and FGF23]. The nCounter PanCancer Pathways Panel assessed gene expression in tumor tissues. Pharmacodynamic variations in serum biomarker levels from baseline were investigated, as were clinical outcome relationships with baseline biomarker levels. They included 407 people in the serum analysis group (lenvatinib $n = 279$, sorafenib $n = 128$) and 58 people in the gene-expression analysis group (lenvatinib $n = 34$, sorafenib $n = 24$). They observed that, whereas both treatments were associated with increases in VEGF, only lenvatinib was associated with increases in FGF19 and FGF23 across the whole study period. Responders had greater levels of FGF19 and FGF23 on cycle 4, day 1 than non-responders (FGF19: 55.2% *vs* 18.3%, $P = 0.014$; FGF23: 48.4% *vs* 16.4%, $P = 0.002$, respectively). In both therapy groups, higher baseline VEGF, ANG2, and FGF21 Levels were associated with a shorter OS. With greater baseline FGF21, lenvatinib had a longer OS than sorafenib [10.9 mo *vs* 6.8 mo, respectively; HR: 0.53; 95%CI: 0.33-0.85; $P = 0.0397$]. In a biomarker examination of tumor tissue, VEGF/FGF-enriched groups outlived the intermediate VEGF/FGF group (HR: 0.39; 95%CI: 0.16-0.91; $P = 0.0253$). They concluded that shorter OS may be predicted by increased baseline levels of VEGF, FGF21, and ANG2. Lenvatinib's superior OS *vs* sorafenib may be predicted by higher baseline FGF21, but further research is required to prove this.

Now, uHCC has an additional first-line MTKI option in lenvatinib. In March 2018, it was approved in Japan. It was also approved in the United States in August 2018. The European Association for the Study of the Liver, European Society for Medical Oncology (ESMO), National Comprehensive Cancer

Network (NCCN), American Society of Clinical Oncology (ASCO), and all agreed that lenvatinib should be used in the first-line setting but only for patients with Child-Pugh class A cirrhosis[49-52].

Combination therapy of immune check-point inhibitor and VEGF inhibitor

Atezolizumab plus bevacizumab: The importance of VEGF and VEGFR signaling pathways in angiogenesis and tumor formation has been highlighted through research into the etiology of HCC. Both healthy and pathological angiogenesis are regulated by the VEGF protein family[53]. When VEGF overexpression was observed in these tumors, their function as therapeutic targets in uHCC was found. It is not unexpected that the idea of targeting tumor angiogenesis as a potential therapeutic method was offered as early as 1971 given the knowledge that a significant phase in tumor development requires oxygen and nourishment supply for sustained growth. As previously stated, VEGF expression is increased in HCC. Sorafenib targets the VEGF signaling system *via* MTKI, resulting in the therapeutic advantage already documented. However, the tangible benefit of sorafenib in terms of VEGF inhibition prompted researchers to look for other pathways targeting tumor angiogenesis and VEGF inhibition.

Because it develops in persistently inflamed livers from both viral and non-viral origins, HCC is usually referred to as an immunogenic malignancy. Furthermore, tumor-associated antigen expression and particular gene alterations that result in unique neoantigens lead to HCC immunogenicity. Innovative immunomodulation treatment options in uHCC have emerged as a result of investigations into the tumor microenvironment (TME) in HCC. Hepatitis viruses' persistent inflammation and the parenchyma's production of cytokines and growth factors coexist in a complex microenvironment. Due to restricted T cell activation that results in the generation of tumor-related antigens, the liver is also known to have an intrinsic immunosuppressive environment. The majority of the immune system's anti-tumor defenses are T cells, and tumor cells that have overexpressed the programmed cell death ligand 1 (PD-L1) have created an immunosuppressive milieu. Immunological checkpoints are coinhibitory membrane glycoproteins that largely block T cell immune overactivation during inflammatory and infectious conditions. Normally, this avoids collateral tissue damage, but in the TME, their expression plays a critical role in encouraging T cell exhaustion and immunological tolerance. The presence of Cytotoxic T Lymphocyte-Associated protein 4 (CTLA-4) and PD-1, immunological checkpoints involved in T cell activation and other inflammatory responses in malignancies, is well understood. In tumor expression on T cell activation and other inflammatory responses, CTLA-4 and PD-1 are immunological checkpoints that have been thoroughly studied. Additionally, expressed on regulatory T cells, CTLA-4 prevents T cells from co-stimulating when antigen is given. This is accomplished by the competitive binding of CD80 and CD86 receptors on antigen-presenting cells, which results in decreased CD28 stimulation and immune escape. As PD-1 binds to its ligands, PD-L1 and PD-L2, the CD28 pathway is also affected, which prevents CD8+ T cell activation and results in immunological inactivation. By expressing PD-L1 and PD-L2, cancer cells use this strategy to escape immune monitoring. Enduring antigen T cell tiredness is caused by exposure to the TME and is demonstrated by an increase in PD-L1 in tumor cells and antigen-presenting cells, which is induced by reactive T cells that express PD-1. As a result, the prognosis is poorer and the tumor grows larger with less effective tumor suppression[54-56].

Bevacizumab, a VEGF monoclonal antibody and atezolizumab, an anti-PD-L1 antibody, have been used in combination therapy for uHCC patients, which has resulted in the most recent and notable advancement in the treatment of HCC[14]. In the TME, VEGF overexpression has been seen to support immunological tolerance and evasion in malignancies. The main function of VEGF is angiogenesis, which paradoxically results in a hypoxic and acidotic TME and attracts immune-suppressive cells like regulatory T cells. VEGF also increases the expression of PD-1 on tumor-infiltrating T cells[57-59]. Targeting VEGF lowers immune suppression, and combining immune checkpoint inhibitors leads in enhanced immunological reactivation *via* increased T cell activity and tumor cell penetration. The phase III IMbrave150 research randomly allocated 501 patients who had not previously undergone systemic treatment to atezolizumab-bevacizumab or sorafenib in a 2:1 ratio. The study found that atezolizumab-bevacizumab improved OS by 67.2% (95%CI: 61.3%-73.1%) at 12 mo while sorafenib improved OS by 54.6% (95%CI: 45.2%-64.0%)[59]. Atezolizumab-bevacizumab had an objective response rate (ORR) of 27.3% (95%CI: 22.5%-32.5%) and sorafenib had an ORR of 11.9% (95%CI: 7.4%-18.0%) according to Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1). This combination's adverse event profile was consistent with the established safety profiles of each medication and the underlying condition. Proteinuria, tiredness, and an increase in aspartate aminotransferase (AST) levels were also observed in 15% of patients with grade 3-4 hypertension. In the atezolizumab-bevacizumab arm, approximately 15% of patients withdrew therapy due to side effects, compared to 10% in the sorafenib arm. The most common reported reason for withdrawal was gastrointestinal side effects. Bleeding is a recognized consequence of bevacizumab due to its anti-angiogenic properties. A typical consequence of cirrhosis is upper gastrointestinal bleeding, which has the potential to be a life-threatening hemorrhage. This was observed in 7% of patients in the atezolizumab-bevacizumab group versus 4.5% in the sorafenib group. Due to the increased risk of catastrophic bleeding with bevacizumab, patients must not have esophagogastric varices prior to beginning treatment. This encouraging advancement has rendered this combination, the FDA-approved preferred strategy for treating uHCC and is currently advised as the first-line treatment in uHCC following the 2021 recommendations[60].

Atezolizumab plus cabozantinib: Kelley *et al*[61] conducted an open-label, randomized, phase 3 study (COSMIC-312) in 837 uHCC patients from 178 centers in 32 countries to compare cabozantinib + atezolizumab against sorafenib as first-line systemic therapy. Patients required to have Child-Pugh class A, ECOG PS 0 or 1, detectable illness as defined by RECIST 1.1, BCLC stage B or C, and adequate organ and marrow function. Through a web-based interactive response system, they were randomly assigned (2:1:1 = 432:217:188) to receive cabozantinib 40 mg once daily plus atezolizumab 1200 mg intravenously (IV) every 3 wk, sorafenib 400 mg twice daily, or cabozantinib 60 mg once daily. Intention to treat (ITT) population had a median follow-up of 13.3 mo, whereas the PFS ITT group had a median follow-up of 15.8 mo (IQR: 14.5-17.2), (IQR: 10.5-16.0). The authors showed that median PFS in the combination therapy arm was 6.8 mo (99%CI: 5.6-8.3) *vs* 4.2 mo (99%CI: 2.8-7.0) in the sorafenib arm (HR: 0.63, 99%CI: 0.44-0.91, P = 0.0012). In the combination treatment group, the median OS was 15.4 mo, compared to 15.5 mo in the sorafenib group (HR: 0.90, 96%CI: 0.69-1.18; P = 0.44). The most frequent grade 3 or 4 AEs included a 9% (38/429) increase in alanine aminotransferase in the combination treatment arm, compared to 3% (6/207) in the sorafenib arm, and 6% (12/188) in the cabozantinib arm. Hypertension was found in 9%, 8%, and 12%, HFS was found in 8%, 8%, and 9%, serious treatment-related AEs occurred in 18%, 16%, and 13% of the combination treatment arm, sorafenib arm, and cabozantinib arm, respectively. Treatment-related grade 5 events occurred in 1% of the combination treatment arm, < 1% of the sorafenib arm, and < 1%) of the cabozantinib arm. They suggested that cabozantinib in combination with atezolizumab might be a therapy option for some uHCC patients[62].

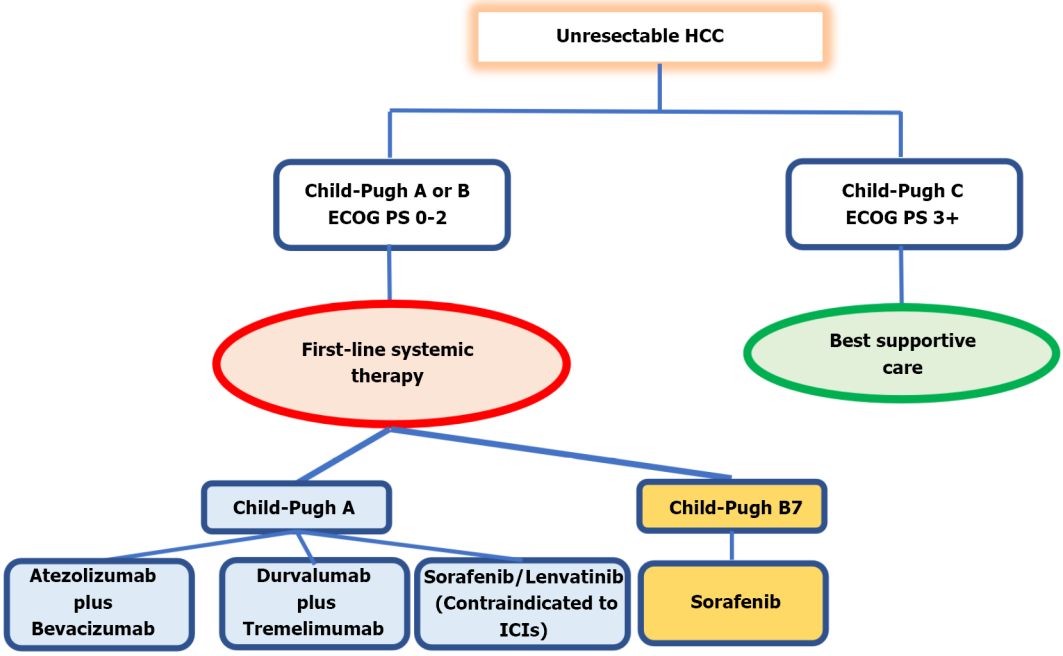
Combination therapy of anti-PD-L1 and CTLA-4 antibody

Durvalumab and tremelimumab: Several studies have shown that extended exposure to CTLA-4 inhibitors may not be required for long-term anti-tumor effects. In metastatic melanoma, Eroglu *et al*[63] found that a single dosage of the CTLA-4 inhibitor tremelimumab might result in exceptionally extended durations of objective anti-tumor responses lasting more than 12 years. In advanced non-small cell lung cancer, a phase Ib research discovered that tremelimumab 1 mg/kg plus durvalumab 20 mg/kg every 4 wk provided an anti-tumor action while being tolerated. In a recent phase I/II study, durvalumab 20 mg/kg with tremelimumab 1 mg/kg every 4 wk for 4 doses was evaluated. The results showed acceptable tolerability and encouraging early efficacy in the second-line setting. The T-300/D-1500 high-dose had the highest risk and benefit outcome with a median OS and ORR of 18.7 mo and 22.7 mo, respectively, in the expanded phase 2 study that examined combinations of 75 mg tremelimumab with 1500 mg durvalumab (T-75/D-1500) and T-300/D-1500. In the phase 3 HIMALAYA study, the T-300/D-1500 group was examined as first-line treatment. Patients with uHCC were divided into four groups: (1) T-300/D-1500 followed by D-1500 every 4 wk (STRIDE); (2) D-1500 every 4 wk; (3) sorafenib 400 mg twice daily; and (4) T-75 every 4 wk followed by D-1500 every 4 wk (T-75/D). T-75/D enrollment was halted due to a planned analysis found no significant difference between D-1500 and T-75/D. D was noninferior to sorafenib alone in terms of OS (16.6 mo *vs* 13.8 mo; HR: 0.86; 96%CI: 0.73-1.03). The T-300/D-1500 ORR was 20.1%, 17% with durvalumab alone, and 5.1% with sorafenib alone. Nevertheless, no statistically significant change in PFS was seen. Durvalumab showed a good safety profile and wasn't worse than sorafenib. Durvalumab generated grade 3/4 AEs in 12.9% of patients treated with the combo group, and sorafenib in 25.8% of patients[64-66]. As a result, the combination of durvalumab plus tremelimumab is a realistic first-line option for patients who are not candidates for atezolizumab or bevacizumab, such as those with a high risk of bleeding[67].

For uHCC patients with a Child-Pugh class A cirrhosis without anticoagulant therapy, an ECOG score of 0 or 1, and treatment for esophageal varices, current ASCO and ESMO guidelines recommend atezolizumab-bevacizumab combination therapy rather than lenvatinib or sorafenib monotherapy. Tremelimumab plus durvalumab is an alternative to bevacizumab for patients who are unsuitable to receive it. Monotherapy with sorafenib or lenvatinib is an alternative treatment for Child-Pugh class B cirrhosis patients with no worse than score 7, or when double immunotherapy is considered unsafe, or when the clinical condition of the patients is less fit, or with multiple comorbidities with predicted poor acceptance to combined immunotherapy. Only individuals with cirrhosis that is no worse than Child-Pugh class A are advised to take lenvatinib. Lenvatinib often causes fewer HFS and alopecia than sorafenib and has a lower overall toxicity profile. According to the REFLECT study, it also had a higher ORR, better PFS, and a longer TTP. As a result, if monotherapy is indicated, the majority of physicians now choose to begin with lenvatinib. However, considering the extended amount of experience and the noninferior median OS observed in the REFLECT study, sorafenib may still be preferable (Figure 1).

Second-line systemic therapy

Single drug-multikinase inhibitor: Regorafenib-Regorafenib was proposed as a possible second-line therapy for uHCC in the RESORCE study in patients who had progressed on sorafenib. The EGFR and VEGF receptors are the targets of the MTKI regorafenib. The phase III, randomized, double-blind trial compared OS to placebo in 567 Child-Pugh class A patients who had been receiving sorafenib and tolerated treatment for at least 20 of the 28 d prior to discontinuation. After enrollment, patients were randomized to receive regorafenib 160 mg daily or a placebo. When compared to the placebo, the median OS with regorafenib was 10.6 mo as opposed to 7.8 mo (HR: 0.63, 95%CI: 0.50-0.79; P < 0.01).



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Figure 1 First-line systemic therapy in unresectable hepatocellular carcinoma. ECOG PS: Eastern Cooperative Oncology Group Performance Status; ICIs: Immune checkpoint inhibitors; HCC: Hepatocellular carcinoma.

Hypertension, hand-foot skin reactions, tiredness, and diarrhea were all grade 3 or 4 AEs. They stated that regorafenib is the only systemic therapy that has been demonstrated to improve survival in HCC patients who are advancing on sorafenib treatment. Regorafenib should be studied in combination with more systemic medications in the future, as well as third-line therapy for patients who do not respond to or tolerate the sorafenib-regorafenib regimen[45].

Cabozantinib-in uHCC patients who had previously been treated with sorafenib and had suffered disease progression on at least one systemic therapy, the MTKI cabozantinib has demonstrated clinical effectiveness with good outcomes. Cabozantinib acts by targeting the VEGF, MET, and AXL receptors. Antiangiogenic resistance, epithelial mesenchymal transition, invasion, and metastasis have all been related to MET and AXL receptors. High levels of MET and AXL expression have been associated with a poor prognosis in HCC, and higher levels of MET activity have been observed in previously treated patients who develop sorafenib resistance. In this double-blind, randomized phase III study (CELESTIAL), 707 patients who had previously taken sorafenib, who had progressed on at least one systemic therapy for HCC, or who had received up to two prior systemic therapies, were randomly assigned to receive cabozantinib 60 mg daily as opposed to a placebo. OS was the main objective, with PFS and ORR as additional endpoints. Patients who received cabozantinib showed a longer OS (10.2 mo *vs* 8.0 mo; HR: 0.76; 95%CI: 0.63-0.92; P = 0.005). When cabozantinib and placebo have been used, PFS was 5.2 mo *vs* 1.9 mo, and ORR was 4% and 1%, respectively. Sixty-eight percent of cabozantinib patients developed grade 3 or 4 adverse effects, with hand-foot skin reactions, hypertension, fatigue, diarrhea, and an increase in liver AST levels being the most frequent. They concluded that in patients with advanced HCC who had already had treatment, cabozantinib therapy produced longer OS and PFS than placebo. Nearly twice as many major side events occurred in the cabozantinib group than they did in the placebo group[68].

Single drug-VEGF inhibitor

Ramucirumab: For the treatment of uHCC patients who have previously had sorafenib therapy and have an AFP of 400 ng/mL or more, ramucirumab has a license. Inhibiting ligand-stimulated VEGFR2, cell proliferation, and angiogenesis, ramucirumab binds to VEGFR2 and blocks the binding of VEGFR ligands VEGF-A, VEGF-C, and VEGF-D. The dose for uHCC patients is 8 mg/kg IV every 2 wk until the condition progresses or there is significant toxicity. For those with mild to moderate hepatic impairment, the manufacturer advises against dose adjustment, but it also notes that clinical deterioration has been observed in patients with Child-Pugh class B and class C cirrhosis who received ramucirumab. Child-Pugh class B or class C cirrhosis patients should only consider ramucirumab if the potential benefits are deemed to exceed the risks of clinical worsening. In a phase 2 trial including 42 uHCC patients, ramucirumab was used as a first-line treatment. Ramucirumab was administered to patients at a dose of 8 mg/kg every 2 wk until the condition progressed or unacceptable toxicity developed. PFS was the primary objective, with response and survival serving as additional goals. The

median PFS, MTP, and survival were each 4.0, 4.2, and 12.0 mo, respectively. The median response time was 14.1 mo, and the disease control rate was 69.0%. There were 4 partial responses (9.5% of patients) [69]. The main ramucirumab trial was the phase 3 REACH trial, which included uHCC patients who were unresponsive to locoregional treatment. All of the patients had previously undergone sorafenib treatment. In addition to best supportive care (BSC), patients were randomly treated with ramucirumab 8 mg/kg IV (n = 283) or a placebo (n = 282) every 2 wk, or until disease progresses, intolerable toxicity, or death. PFS, response, and disease control were secondary goals, with OS serving as the primary objective. The median survival time with ramucirumab was 9.2 mo compared to 7.6 mo with placebo (P = 0.14). In the ramucirumab and placebo groups, the median PFS times were 2.8 mo and 2.1 mo, respectively (P < 0.01). MTP for ramucirumab was 3.5 mo and 2.6 mo for the placebo (P < 0.01). In comparison to the placebo group, which only saw two partial responses (1% of patients), the ramucirumab group showed a full resolution and 19 partial responses (7% of patients) (P < 0.01). Fifty six percent of ramucirumab-treated individuals and 46% of placebo receivers achieved disease control (P = 0.011). The reductions of dosage were required in 7% of patients who were treated with ramucirumab and less than 1% of placebo patients, with dose omission rates of 22% and 10%, respectively; 10% and 3% of patients in the ramucirumab and placebo groups were discontinued due to unfavorable drug side effects. The most frequent grade 3 or 4 AEs were ascites (5% of patients who were treated with ramucirumab *vs* 4% of placebo patients), AST elevation (5% *vs* 8%), thrombocytopenia (5% *vs* 1%), hypertension (12% *vs* 4%), asthenia (5% *vs* 2%), and hyperbilirubinemia (1% *vs* 5%). Investigations were conducted on a predetermined sample of individuals having a baseline AFP level of 400 ng/mL. With ramucirumab, the median survival time was 7.8 mo as opposed to 4.2 mo with a placebo (P = 0.006). Survival was 11.8 mo with placebo and 10.1 mo with ramucirumab in patients with an AFP value less than 400 ng/mL (P = 0.51). These results led the researchers to propose that a high baseline AFP level may be a predictor of who may respond favorably to ramucirumab treatment[70].

The objective of the phase 3 study (REACH-2) was to address the efficacy of ramucirumab treatment in baseline AFP levels of 400 ng/mL or above patients. They were recruited because they had an ECOG PS of 0 or 1, intolerance to sorafenib, Child-Pugh class A cirrhosis, a history of disease progression, and they weren't candidates for locoregional therapy or resistant to it. Every 2 wk, patients were treated with ramucirumab 8 mg/kg (n = 197) or a placebo (n = 95), along with BSC, until the illness progressed or the side effects became unbearable. When ramucirumab was used instead of a placebo, the response rates were 5% and 1%, respectively (P = 0.1697), while the rates of disease control were 59.9% and 38.9%, respectively (P < 0.001). When compared to placebo, treatment with ramucirumab was related with significantly prolonged median PFS (2.8 mo *vs* 1.6 mo, P < 0.001) and median survival (8.5 mo *vs* 7.3 mo, P = 0.019). The REACH-2 study revealed that ramucirumab gave significantly better survival than placebo when taken as follow-up treatment following sorafenib in patients with an AFP value of 400 ng/mL or more. Ramucirumab did not cause HFS, a well-known side effect of other targeted medications[71]. Post hoc analysis of the REACH and REACH-2 studies confirmed the relevance of AFP as a predictive indicator, with AFP response considerably greater in individuals treated with ramucirumab than placebo (P < 0.0001). Survival was considerably enhanced with an AFP response (13.6 mo *vs* 5.6 mo; HR: 0.45; P < 0.0001)[72]. Ramucirumab is advised by NCCN as a category 1 medication for further therapy following sorafenib usage in individuals with an AFP level of 400 ng/mL or more[52].

Single drug-immune check-point inhibitor

Pembrolizumab: In the phase II KEYNOTE-224 research, pembrolizumab, an anti-PD1 monoclonal antibody, demonstrated comparable clinical antitumor effectiveness and safety when given to patients who had previously taken sorafenib for uHCC patients[11]. Finn *et al*[13] performed a phase 3 randomized, double-blind trial at 119 healthcare centers in 27 countries. With a follow-up length of 13.8 mo for pembrolizumab and 10.6 mo for placebo, they randomly allocated 413 patients to receive pembrolizumab plus BSC or placebo plus BSC. The median OS for pembrolizumab was 13.9 mo compared to 10.6 mo for placebo. At the end of the study, the median PFS for pembrolizumab was 3.0 mo *vs* 2.8 mo for placebo. Pembrolizumab was used in 147 (52.7%) and 62 (46.3%) patients, respectively; treatment-related events occurred in 52 (18.6%) and 10 patients (7.5%), respectively. No new cases of hepatitis C or B were discovered. They concluded that OS and PFS did not satisfy the criteria for statistical significance. Pembrolizumab offers a positive risk-to-benefit ratio in this population, according to the results, which are similar to those of KEYNOTE-224.

Drug-combination of PD-1 and CTLA-4 antibody

Nivolumab and ipilimumab: The CTLA-4 molecule, a crucial signaling checkpoint required for T-cell activation, is the target of the immune check-point inhibitor (ICI) ipilimumab. They successfully target two different immunological checkpoints when combined with nivolumab, inducing the modify immune response. The United States FDA approved nivolumab with ipilimumab as a second-line therapy in March 2020. The efficacy of combination treatment was proven in the phase 1/2 CHECKMATE-040 trial, which included 148 patients who were treated with sorafenib and clinically were better than Child-Pugh class A. The study evaluated 3 different regimens: Arm A: Nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every 3 wk for 4 cycles, followed by biweekly nivolumab 240 mg;

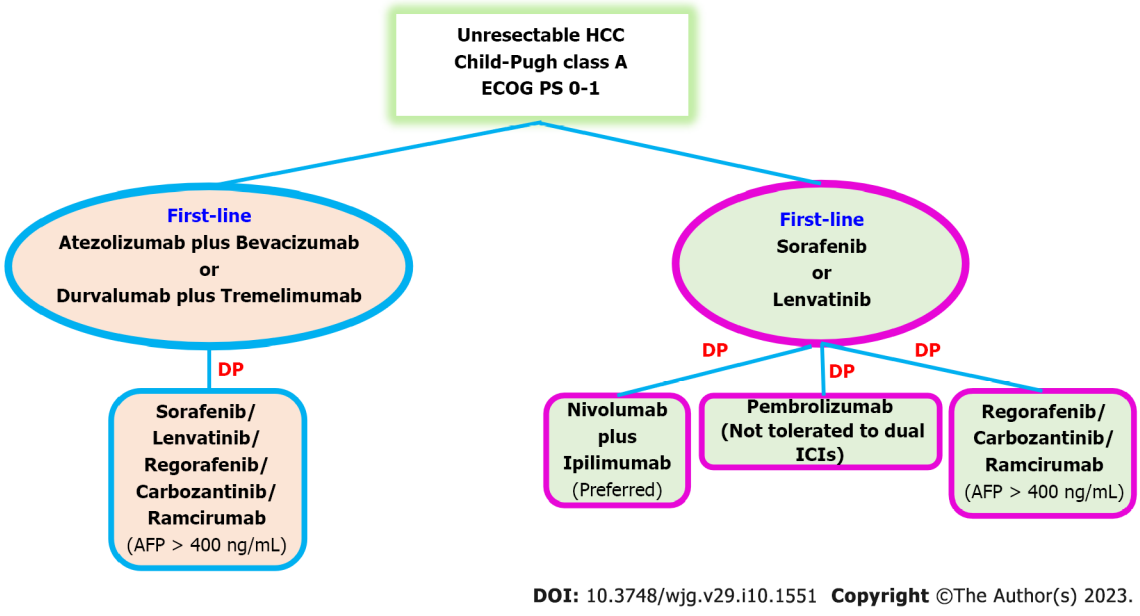
Arm B: Nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 wk for 4 cycles, followed by biweekly nivolumab 240 mg; Arm C: Nivolumab 3 mg/kg every 2 wk plus ipilimumab 1 mg/kg every 6 wk. The suggested regimen is Arm A. According to the findings, the proposed regimen had the best ORR of 32%. CR was obtained by 8% of patients, and PR by 24%. The duration of response was 17 mo on average. The disease control rate was comparable among the three groups. However, larger sample size experiments are required to corroborate this conclusion[73]. In this CHECKMATE-040 cohort, individuals with or without hepatitis B or C had similar patterns of AEs, however Arm A was associated with higher TRAEs. Due to TRAEs, treatment was stopped in 18% of Arm A patients, 6% of Arm B patients, and 2% of Arm C patients. Rashes, adrenal insufficiency, hypothyroidism or thyroiditis, colitis, pneumonitis, and infusion-related complications were all observed in 35%, 18%, 22%, 10%, 10%, and 8% of patients, respectively.

In conclusion, the appropriate second-line therapy regimen and sequencing are not well established and rely on the patient's PS, liver function, and choice of first-line therapy. TKIs like sorafenib, lenvatinib, or cabozantinib are suggested as second-line treatments for patients who have previously received atezolizumab plus bevacizumab or durvalumab plus tremelimumab. Given the possibility for a greater ORR than single medications, combination immunotherapy with nivolumab-ipilimumab is preferred for patients who have become worse on TKIs such sorafenib or lenvatinib. Pembrolizumab is an alternative if the patients are unable to take double ICIs. Regorafenib or cabozantinib may be considered as second-line options if sorafenib or lenvatinib have been chosen as the first-line therapy and the patients have a contraindication to ICIs. Patients with an AFP level > 400 ng/mL are advised to use ramucirumab[74] (Figure 2).

FUTURE TRENDS IN SYSTEMIC COMBINATION THERAPY

Since the rapid FDA approval of atezolizumab and bevacizumab, combining checkpoint inhibitors with multikinase inhibitors-especially anti-angiogenesis therapy-has gained widespread acceptance. Numerous studies have shown that the synergistic interaction of ICIs and TKIs promotes vascular remodeling and tumor immune activation[75-77]. In a phase Ib study, lenvatinib and pembrolizumab were investigated. The median PFS, OS, and ORR for this combination were 9.7 mo, 20.4 mo, and 46%, respectively, showing substantial anti-tumor effectiveness. Most of the AEs might be controlled by changing the dosage[78]. The combination of camrelizumab, an anti-PD-1 antibody, and apatinib, an orally active VEGFR-2 inhibitor, was also investigated in a dose-expansion and escalation phase I research. The suggested dosage of camrelizumab 200 mg every 2 wk with apatinib 250 mg daily showed therapeutic advantages with a 50% ORR. This regimen was thus investigated in the phase 2 RESCUE study. For the treatment of naïve uHCC patients or those who had previously failed or were intolerant to TKIs, apatinib 250 mg was administered orally every day combined with camrelizumab 200 mg IV (body weight > 50 kg) or 3 mg/kg (body weight 50 kg) every 2 wk. A total of 70 patients and 120 patients, who were mainly HBV-infected (88.3%) in the first-line and second-line settings, respectively, were included. The median time since the cutoff for the data was 29.1 mo. The 2-year OS was 43.3% and the median OS was 20.1 mo in the first-line setting. The median OS in the second-line condition was 21.8 mo, with a 2-year OS of 44.6%. A phase 3 study is underway to evaluate its effectiveness in the first-line situation compared to that of sorafenib[79].

To assess the effectiveness and tolerability of lenvatinib plus camrelizumab *vs* lenvatinib monotherapy as first-line therapy, Li *et al*[80] performed a multicenter, retrospective cohort investigation of 92 uHCC patients. In contrast, 44 patients received oral lenvatinib 12 mg or 8 mg daily and 48 patients received intravenous camrelizumab 200 mg every 3 wk. The ORR in the combination group was shown to be significantly higher than in the monotherapy group (RECIST 1.1: 37.5% *vs* 13.6%, P = 0.009). The median OS in the monotherapy group was 13.9 mo (95%CI: 13.3-18.3), but not in the combination group (P = 0.015). Lenvatinib with camrelizumab had a 1-year survival rate of 79.2%, compared to lenvatinib monotherapy's 56.8%. Lenvatinib with camrelizumab had a significantly longer median PFS than lenvatinib monotherapy (10.3 mo *vs* 7.5 mo, P = 0.009). Subgroup analysis revealed that combination therapy was associated with a longer OS in males, patients with a Child-Pugh score < 7, patients with three or more tumors, patients with AFP levels greater than 200 ng/mL, HBV-positive patients, patients with vascular invasion, and patients without hypertension. In the lenvatinib plus camrelizumab and lenvatinib monotherapy groups, the AEs that affected more than 20% of patients were HFS (22.9% *vs* 25.0%, P = 0.81), hypertension (33.3% *vs* 38.6%, P = 0.59), diarrhea (31.2% *vs* 31.8%, P = 0.95), and loss of appetite (41.7% *vs* 40.9%, P = 0.90). There were no statistically significant variations in the occurrences of any AEs between the two groups. They concluded that first-line lenvatinib with camrelizumab treatment may benefit patients with uHCC better than lenvatinib alone. There were no new safety signals, and the toxicity and tolerance profiles of the two treatment protocols appeared to be comparable. Further research is necessary before deciding if lenvatinib and camrelizumab treatment together can provide uHCC patients a unique therapeutic alternative.



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Figure 2 Second-line systemic therapy in unresectable hepatocellular carcinoma. DP: Disease progress; ECOG PS: Eastern Cooperative Oncology Group Performance Status; ICIs: Immune checkpoint inhibitors; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein.

CONCLUSION

Systemic therapy for HCC has significantly advanced with a major breakthrough since the FDA approved sorafenib in 2007. Although several MTKIs have shown promise in the therapy of uHCC, the discovery of ICIs has completely changed the field, bringing about remarkable ORR and OS benefit. This is demonstrated by the innovative outcomes from the HIMALAYA trial, the IMbrave150 study, and the CHECKMATE-040 trial for the second-line combination of nivolumab-ipilimumab, atezolizumab-bevacizumab, durvalumab-tremelimumab, and atezolizumab-bevacizumab, respectively. Each of the aforementioned regimens has a somewhat distinct toxicity profile, and combination therapy is correlated with greater toxicities. In clinical practice, single-agent therapies are explored for patients who are less fit or have severe medical comorbidities, whereas combination treatments are studied for very fit patients, such as those with a status of performance 0 to 1 and no importance medical comorbidities. Additionally, an esophago-gastro-duodenoscopy must be performed before starting atezolizumab-bevacizumab therapy in order to address any esophageal varices due to the relatively high dose of bevacizumab used-15 mg/kg-which is associated with a higher bleeding risk.

Because of this, it is still unclear which patient groups may benefit from or tolerate a certain combination of therapies better than others. Additionally, there are very few clinical trials investigating the function of future therapy in individuals who respond to ICIs. This topic is being actively investigated by a number of current studies, including a phase II study looking at cabozantinib in the post-ICI situation and other studies comparing the combination of atezolizumab-lenvatinib to sorafenib in the post-atezolizumab-bevacizumab scenario. Recently, anti-PD-1 medication has been controversially used in patients with non-alcoholic steatohepatitis-related HCC (NASH-HCC)[81]. In addition, Pfister *et al* [82] examined anti-PD-1 therapy animal models with NASH in both therapeutic and preventative contexts. They demonstrated that anti-PD-1 therapy accelerated hepatocarcinogenesis and produced hepatic fibrosis in NASH-mice without tumors. Treatment with anti-PD-1 did not result in tumor regression in NASH-mice carrying HCC. Surprisingly, it has, on the contrary, sped up the tumor's growth. They conducted a meta-analysis of 1656 patients from 3 significant studies to determine whether similar outcomes were also observed in human HCC (CHECKMATE-459, IMBrave150, and KEYNOTE-240). In accordance with the underlying etiology of HCC, they also evaluated the survival results of HCC treated with immunotherapy. Individuals with viral HCC had a longer survival time with immunotherapy (HR: 0.64; 95%CI: 0.48-0.94), but those with non-viral HCC did not (HR: 0.92; 95%CI: 0.77-1.11). According to a research, non-viral HCC patients who received anti-PD-1 medication had the same ORR and PFS as patients with viral HCC. Alternative explanations have been developed in response to these seemingly incongruous findings, including the varied population with non-viral HCC and the dearth of knowledge surrounding follow-up therapies[83]. It's also essential to remember that these worse outcomes for immunotherapy-treated non-viral HCC patients were retrospectively determined. As a result, based on the etiology of HCC, this cannot result in a shift in clinical management for uHCC patients. To clarify these concerns, more clinical studies with predetermined stratification should be planned.

Unanswered questions about therapeutic drug resistance and predictive biomarkers still exist. Drug resistance is prevalent and is generally believed to be the leading cause of therapeutic failure. EGFR activation, the existence of cancer stem cells, tumor-initiating cells, and the epithelial-mesenchymal transition (EMT) are examples of potential pathways. Cancer stem cells are created when EMT signals are activated, and these cancer stem cells have the ability to self-renew and differentiate. In human liver cell lines, prolonged sorafenib exposure results in EMT, which is characterized by changes in cell shape, loss of E-cadherin, and elevated vimentin expression[84,85]. In a genetically engineered mouse model of HCC, it was discovered that β-catenin activation encourages immune evasion and resistance to PD-1 inhibitors[86]. Although additional study is required to translate this bench-to-bedside data, these biomarkers have the potential to affect treatment choices. Our knowledge of tumor biology is still lacking, and there is an unmet need for cutting-edge drugs to deal with these resistance mechanisms. To accurately predict prognosis and the therapeutic response to target treatment or immunotherapy, there are yet no meaningful molecular indications. In actuality, imaging methods are used to identify the majority of HCCs, and the available treatments are much the same. It makes sense to look at biomarkers that might inform treatment choices in the era of personalized medicine. The most extensively researched immunotherapy biomarkers, including as tumor mutational burden, microsatellite instability, and PD-L1 expression, are presently employed very seldom in uHCC[87]. Although it has been shown that circulating markers including AFP, tumor necrosis factor-alpha, and IL-6 associated with HCC treatment outcomes, further studies are required to confirm these findings. A gene expression test has also recently been investigated as a possible biomarker for predicting immunotherapy response[88].

Ultimately, more research is necessary to determine any potential indirect drug interactions between antibiotics, proton pump inhibitors, ICIs, or multiple therapies. It is known that administering immunotherapy and antibiotics at the same time has a detrimental impact on the effectiveness of anti-cancer treatment. Numerous studies have shown the close relationship between ICIs and gut flora. Intestinal homeostasis and the reduction of systemic inflammation are crucially dependent on the microbiota. Antibiotics have a deleterious effect by causing dysbiosis, which changes the immune system's systemic anti-tumor response during, or in the 1st few weeks before commencing ICIs[89].

Since sorafenib was the only first-line therapy option for uHCC for 15 years, three alternative first-line therapeutic options have emerged. This represents a significant improvement in the management of uHCC (atezolizumab-bevacizumab, sorafenib, and lenvatinib). Cabozantinib, regorafenib, and ramucirumab are now included in the second-line therapy alternatives (AFP > 400 ng/mL). Nivolumab and pembrolizumab, ICIs, have been suggested as second-line treatments for individuals who are intolerant to MTKIs.

FOOTNOTES

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MINIREVIEWS

Systemic treatment for metastatic colorectal cancer

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Abstract

Significant progress has been achieved in the treatment of metastatic colorectal cancer (mCRC) patients during the last 20 years. There are currently numerous treatments available for the first-line treatment of mCRC. Sophisticated molecular technologies have been developed to reveal novel prognostic and predictive biomarkers for CRC. The development of next-generation sequencing and whole-exome sequencing, which are strong new tools for the discovery of predictive molecular biomarkers to facilitate the delivery of customized treatment, has resulted in tremendous breakthroughs in DNA sequencing technology in recent years. The appropriate adjuvant treatments for mCRC patients are determined by the tumor stage, presence of high-risk pathologic characteristics, microsatellite instability status, patient age, and performance status. Chemotherapy, targeted therapy, and immunotherapy are the main systemic treatments for patients with mCRC. Despite the fact that these novel treatment choices have increased overall survival for mCRC, survival remains optimal for individuals with non-metastatic disease. The molecular technologies currently being used to support our ability to practice personalized medicine; the practical aspects of applying molecular biomarkers to regular clinical practice; and the evolution of chemotherapy, targeted therapy, and immunotherapy strategies for the treatment of mCRC in the front-line setting are all reviewed here.

Key Words: Systemic treatment; Metastatic colorectal cancer; Personalized medicine; Biomarkers; Chemotherapy; Targeted therapy; Immunotherapy

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Core Tip: Advances in the molecular profiling of metastatic colorectal cancer allow treatment to be tailored to the biologic characteristics of the tumor for certain patient subgroups. Although cures are still rare, more people can expect to live longer. Genomic profiling enables therapy selection, allowing more individuals to benefit while exposing fewer to the harm of ineffective medicines. An important component in determining treatment results is the choice of an effective first-line therapy, which should consider both clinical considerations and molecular indicators. The systemic treatments used in the first-line regimen determine the second-line regimen. Third-line therapy, which includes epithelial growth factor receptor inhibitors for patients with rat sarcoma virus wild-type, should consider molecular profiling. Patients with high microsatellite instability illnesses may be candidates for immunotherapy with pembrolizumab or nivolumab plus ipilimumab.

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INTRODUCTION

The third most commonly occurring cancer in humans is colorectal cancer (CRC). More than 2 million individuals are diagnosed with this cancer each year worldwide. This year, about one million individuals will die from CRC. The liver is the most common target of CRC hematogenous metastasis, as well as the site most responsible for death from this common malignancy. When patients are diagnosed in the late stage of disease, their prognosis remains poor[1,2]. The majority of individuals who have a CRC diagnosis are over 50, whereas only 12% of all new CRC diagnoses are found in those under 50. Overall, the lifetime risk of developing CRC is about 1 in 23 (4.3%) for men and 1 in 25 (4.0%) for women. According to statistics from the Surveillance, Epidemiology, and End Results Program, at the time of diagnosis, 38% of patients have localized disease, 35% have regional disease, 21% have distant disease, and 6% have no stage[3]. CRC diagnoses have decreased overall since 2000 as a result of increased screening efforts, although it has increased in young people under 50 since the 1990s. Preventive measures, such as routine colonoscopies, remain the most effective way to combat CRC. With a rising interest in non-invasive biomarkers, many additional approaches have been developed. However, nearly 50% of patients are still diagnosed at an advanced stage. Metastatic CRC (mCRC) has a poor prognosis with a 5-year survival rate of 14%, and this number has remained constant over the past 5 years. Until the late twentieth century, mCRC was thought to always be fatal. The discovery that metastatic cancer is mostly localized to the liver in postmortem investigations and radiologic examinations utilizing computer-assisted tomography scanners led early pioneers to resect liver metastasis. To establish liver resections for metastatic carcinoma as an acceptable treatment, pioneers conducted and published data[4]. Only one standard regimen to manage CRC has been shown to be inefficient, resulting in high rates of treatment failure and disease resistance. Recently, the clinical outcomes of patients with mCRC have improved dramatically as a result of the discovery of prognostic and predictive molecular biomarkers and the subsequent individualization of treatment options. High genetic heterogeneity, including but not limited to chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP), has been identified by molecular profiling of CRC. Different CRC subtypes have varying prognoses and therapeutic outcomes. Promising improvements for the use of systemic therapy and precision medicine in mCRC have resulted from recent advancements in our understanding of the molecular signaling networks that control intestinal regeneration and homeostasis[5].

The discovery of the key molecular drivers in CRC pathogenesis, coupled with the ability to screen tissue for measurable mutations crucial to disease progression, led to the development of an innovative treatment model for patients with advanced CRC. Despite substantial breakthroughs in tumor biology understanding, these have not entirely translated into proven novel therapies for all patients, since the chemotherapeutic strategy is still built around combination cytotoxic regimens targeted at proliferative epithelium. Although there have been some achievements with targeted therapy, such as the synergistic effect of protein kinase B-Raf (BRAF) inhibitors and epithelial growth factor receptor (EGFR) inhibitors in *BRAF*-mutant CRC, certain medications have not been able to offer clinically meaningful improvements for other pathways. As evidenced by the effect of immune checkpoint inhibition in microsatellite unstable CRC, therapeutic exploitation of intercompartmental signaling in the malignant epithelium may represent an important new drug paradigm in CRC. This is because we are becoming more aware of the signaling crosstalk that controls intestinal cell fate in health and disease, as well as the function of the tumor microenvironment (TME)[6,7].

We have split these important signaling pathways into those that control the destiny of cancer cells or intestinal epithelium directly and those that function indirectly by leveraging the TME in this review. Additionally, we explore how signaling affects each cancer cell's destiny and comment on some possible treatment prospects that result from effective pathway modulation for each.

RISK FACTORS FOR CRC

Behaviors, diets, and lifestyle

Only a small proportion of CRCs are associated with germline mutations or discovered in the presence of a strong family history; the majority of CRCs are random[8,9]. The significance of environmental exposure has been further demonstrated by the variation in CRC risks throughout the world and the discovery that younger generations are at a higher risk of CRC in westernized nations. Numerous studies have been conducted in an effort to identify and quantify the environmental and dietary risk factors for CRC. Global studies have shown a 45-fold variation in the age-standardized incidence of CRC worldwide[10]. Gambia and other non-industrialized nations have the lowest rates of CRC, whereas westernized nations have the highest rates. The prevalence of CRC has been seen to rise over time when a nation industrializes and starts to follow a westernized lifestyle and diets low in fiber[11,12]. A westernized diet, or one that is low in fiber, fruits, and vegetables and heavy in processed meats, sugary drinks, and refined grains, is linked to greater risks of CRC. It has been challenging to pinpoint everything that increases the risk of CRC in a westernized diet. This diet's many components are probably a factor in the greater prevalence of CRC. Studies have repeatedly shown that diets rich in processed foods and red meat are linked to higher risks of CRC. For every 100 g of red and processed meat consumed, CRC incidence increases by 12%, according to a recent meta-analysis of 111 studies involving 400 individuals[13] (Table 1). Smoking increases the likelihood of both serrated polyps and colorectal adenomas[14]. According to large observational studies, more pack-years result in higher CRC rates[15]. Recent research suggests that smoking is marginally related to MSI-high (MSI-H) tumors, increases the rates of rectal and proximal CRC, and is correlated with *BRAF*-mutant malignancies. Similar to smoking, drinking alcohol is a recognized risk factor for CRC, and recent pooled studies have demonstrated that even occasional drinking increases the risk of CRC[16].

Numerous studies have shown that an increased risk of CRC is related to obesity and decreased physical activity. CRC has repeatedly proven to be correlated with excess body fat, which is most typically quantified using body mass index and waist circumference. Sedentary activity, such as extended sitting or TV viewing, is linked to a higher risk of CRC. In populations over 50, the majority of research verifying obesity and a lack of physical exercise as risk factors for CRC has been demonstrated. Attempts to quantify the impact of obesity and physical activity on rates of early-onset CRC have rekindled attention in light of the rising burden of young-onset CRC in developed nations[17,18]. These and other investigations support the advice from the American Cancer Society and other cancer organizations that maintaining a healthy weight and engaging in more physical exercise are crucial for lowering the risk of CRC.

Genetic factors

A family history of cancer without a specific condition is thought to be the cause of 25% of CRCs, while hereditary cancer syndromes are thought to be the cause of 5% of CRCs. The hereditary component of CRC is predicted to be 35%-40%. Hereditary non-polyposis CRC (Lynch syndrome), familial adenomatous polyposis (FAP), and MUTYH-associated polyposis (MAP) are the most prevalent hereditary cancer syndromes. About 2%-4% of all CRCs are caused by Lynch syndrome, the most prevalent hereditary CRC condition. Patients with Lynch syndrome are susceptible to endometrial, ovarian, stomach, small intestine, hepatobiliary tract, pancreatic, ureter, and renal pelvis malignancies. Lynch syndrome is caused by mutations in the DNA mismatch repair genes *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2*[19,20]. The lifetime risk of CRC for these people is between 5% and 85%. FAP makes up roughly 1% of CRC cases and is the second most prevalent hereditary CRC syndrome. A germ line mutation in the adenomatous polyposis coli (*APC*) gene that causes a shortened APC protein is the secondary cause of FAP. With a 90% inheritance, it is inherited in an autosomal dominant manner. For these people, thousands of polyps form in the gastrointestinal system, the majority of which are in the colon. By the third or fourth decade, CRC will manifest in all FAP-affected individuals without preventative colectomy[21]. The lifetime risk of CRC for these MAP individuals is expected to be 43%-100%. Serrated polyposis syndrome, Peutz-Jeghers syndrome, and juvenile polyposis syndrome are further polyposis syndromes associated with a higher risk of CRC. These are all uncommon disorders that together only contribute to 1% of CRC incidence. Race, age, and sex are other unmodifiable risk factors for CRC in addition to family history. Male patients are generally at a higher risk of developing CRC than female patients; ideas explaining this include the fact that women typically have less visceral fat and benefit from estrogen's general preventive properties against CRC. Men are also less likely to pursue screening, and they may be more exposed to environmental risks, including drinking, smoking, and unhealthy diets[22].

Table 1 Modifiable and non-modifiable risk factors and preventive factors for colorectal cancer

	Modifiable	Non-modifiable
Risk factors	Excess body fat	Family history of CRC
	Sedentary life-style	Advanced polyps
	Westernized diet	Polyposis syndrome (FAP, MAP)
	Processed meats	Lynch syndrome (HNPCC)
	Red meats	Black people
		Older age
		Male
Preventive factors	High fiber diet	Female
	Whole grain diet	
	No alcohol	
	No smoking	

CRC: Colorectal cancer; FAP: Familial adenomatous polyposis; HNPCC: Hereditary non-polyposis colorectal cancer; MAP: MUTYH-associated polyposis.

Polyps

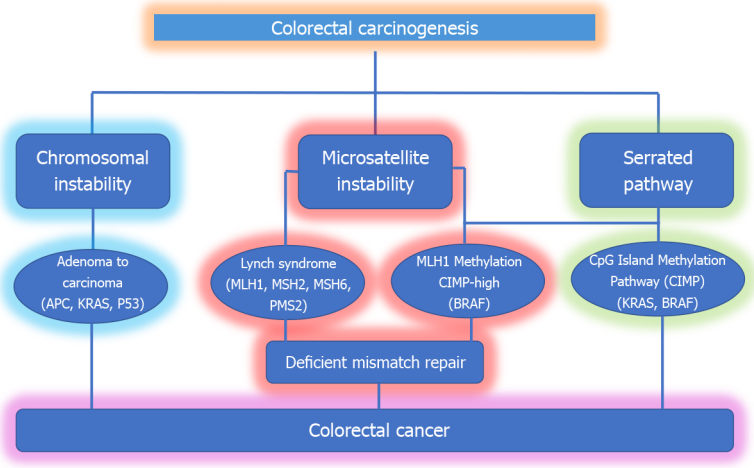
Most CRCs develop from a harmless precursor polyp. Therefore, people who have a considerable personal or family history of high-risk polyps are at a higher risk of developing CRC. There are many different forms of polyps, some of which are non-neoplastic, including hyperplastic polyps, mucosal polyps, inflammatory polyps, and harmartomatous polyps. The remaining polyps, including adenomatous and serrated polyps, have cancerous potential. Adenomatous polyps are where the vast majority of sporadic CRCs originate. By the time they are 50, roughly one-third of individuals are predicted to develop polyps. However, the majority of these will not progress to CRC. Villous histology, high-grade dysplasia, and polyps larger than 1 cm all enhance the likelihood that they may develop into CRC. Serrated polyps are believed to be antecedents for up to 10%-15% of sporadic CRCs, albeit less frequent[23,24].

COLORECTAL CARCINOGENESIS

Three molecular pathways have been hypothesized for colorectal carcinogensis, two of which center on the growth of polyps into cancerous tumors. The traditional pathway depicts the long-term evolution of normal cells to adenomas and finally to carcinomas (the adenocarcinoma sequence). This causes 85%-90% of all sporadic CRCs and is mostly related to the growth of tumors that are CIN. It is frequently accompanied by an early *APC* gene mutation, activation of the growth-promoting oncogenes Kristin rat sarcoma virus (*KRAS*) or *BRAF*, and additional mutations that cause cancer to proceed. About 10%-15% of sporadic CRCs are caused by the CIMP/serrated pathway[25,26]. Due to these changes in the methylation of gene promoter regions and general hypomethylation, many genes are silenced. Early BRAFV600E mutations are frequently seen in these tumors, which activate the mitogen-activated protein kinase (MAPK) pathway and cause the growth of hyperplastic polyps[27]. The MSI-H pathway is the third route. More than 95% of the malignancies related to Lynch syndrome are caused by this important pathway. Outside of Lynch syndrome, MSI is a rare condition that is caused by a lack of DNA mismatch repair genes, which eventually results in altered DNA sequences (Figure 1).

KRAS

A proto-oncogene called *KRAS*, which produces a GTPase protein, is essential for intracellular signal transduction downstream of membrane-bound receptors like EGFR. Uncontrolled cell growth, proliferation, survival, migration, and invasion ensue from a mutation in *KRAS* because it causes constitutive activation of the MAPK pathway, regardless of independent activation of the upstream EGFR receptor. Activating *KRAS* mutations have been reported in a variety of cancers and have been found between 30% and 50% of CRC patients[28,29]. *KRAS* is a crucial biomarker for prognosis and prediction in the management of mCRC. *KRAS*-mutated tumors in mCRC have a poorer prognosis and are more likely to exhibit aggressive biology, spread metastatically, recur, and result in mortality. *KRAS* mutation is independently linked to poorer progression-free survival (PFS) and overall survival (OS) in individuals who underwent hepatic resection for mCRC, according to data from those patients. *KRAS* codon 12 has been linked to lower recurrence-free survival in variations of *KRAS* mutations throughout all stages of



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Figure 1 Three molecular pathways can lead to colorectal cancer. For colorectal carcinogenesis, three molecular pathways have been proposed: the chromosomal instable or classic pathway, the microsatellite instability pathway, and the serrated pathway. APC: Adenomatous polyposis coli; BRAF: Protein kinase B-Raf; CIMP: CpG island methylator phenotype; KRAS: Kristin rat sarcoma virus.

mCRC[30]. Data suggest that curative resection may not be advantageous in the metastatic scenario in *KRAS* mutant patients who also have other poor clinical prognostic characteristics, such as node-positive disease or extensive metastases, due to the prognostic consequences of *KRAS* mutations[31]. Additionally, the existence of a *KRAS* mutation acts as a biomarker for the therapeutic efficacy of some therapies, such as EGFR inhibitor therapy. Anti-EGFR treatment has been shown to be effective in treating KRAS wild-type (WT) cancers in several clinical studies. Due to the independent constitutive activation of KRAS downstream of EGFR, which persistently encourages cell growth and division, anti-EGFR treatment is not helpful in patients with *KRAS* mutations[32]. Although EGFR inhibition is effective in treating the majority of KRAS WT tumors, some patients continue to have resistance, necessitating more research. Mutations in other RAS family oncogenes, such as neuroblastoma RAS (NRAS) and Harvey RAS, identify tumors that are resistant to anti-EGFR treatment. Other phosphorylation pathway genes that work downstream of EGFR, such as phosphoinositide 3-kinases, phosphatase and tensin homolog, MAPK, and mitogen-activated protein kinase kinase (MEK), have not been demonstrated to be accurate predictors of the EGFR response for mCRC, and research into the causes of anti-EGFR resistance in this patient group is still underway[33].

BRAF

BRAF, a serine/threonine-protein kinase, is an essential component of the MAPK signaling cascade downstream of KRAS. In less than 10% of CRC patients, *BRAF* mutations have been found. The same MAPK pathway that KRAS uses for BRAF signaling also uses it, and functional mutations in either of these genes have identical effects on phenotypic and treatment implications. As a predictive biomarker for mCRC, the *BRAF* mutation is linked to worse outcomes, shorter survival, and a greater incidence of peritoneal and distant lymph node involvement. In patients with mCRC undergoing curative-purpose hepatectomy, the *BRAF* mutation is associated with poorer survival compared to both *BRAF* WT and *KRAS* mutated tumors[34,35]. BRAF also functions as a prognostic marker. Vemurafenib, a direct BRAF inhibitor, was first discovered through early attempts at targeted medication treatment for melanoma. Studies have been conducted to determine if BRAF inhibition has comparable effects on CRC. BRAF inhibition and EGFR inhibitors together produced an OS improvement. In patients with *BRAF* mutant mCRC, encorafenib in conjunction with EGFR inhibition is a potent form of newer generation BRAF inhibition therapy[36].

Human epidermal growth factor 2

A further promising target for mCRC is human epidermal growth factor 2 (HER2), which is essential for intracellular signal transduction. HER2 plays a role in the development of breast cancer, and trastuzumab and other HER2 inhibitors can be used to specifically treat the disease. Because there are so many targeted treatments available, many researchers have concentrated on HER2 mutations in the mCRC population, despite the fact that only a small percentage of patients (10%) overexpress HER2. Trastuzumab with pertuzumab, trastuzumab with lapatinib, and fam-trastuzumab deruxtecannxki are currently available as HER2 inhibitors. Even though it only accounts for a small proportion of all mCRC patients, the HER2 amplified condition serves as a predictive biomarker for HER2 targeted therapy with the potential for a therapeutic response[37-39].

SYSTEMIC TREATMENT FOR MCRC

A surgeon may be able to completely remove a few metastatic foci of mCRC, which are often located in the liver or lung. When the main tumor and all metastases can be completely removed surgically, mCRC is said to be resectable. However, nodal infiltration and covert micrometastatic spread are frequent in these individuals. Less than 20% of individuals with mCRC who undergo resection are permanently cured. Oncologists from surgical and medical branches should work together to develop treatment strategies when mCRC may be resectable. If the main tumor is in the rectum, radiation oncologists should be consulted. The main therapy for mCRC is systemic chemotherapy. The United States Food and Drug Administration (FDA) has authorized many medications for the treatment of mCRC. However, for the majority of patients, mCRC is still incurable. Despite the rarity of a cure for mCRC, recent major clinical trials with patients who could tolerate chemotherapy have demonstrated that patients can live for 2 to 3 years with intense treatment and numerous systemic medicines. Survival is influenced by the molecular subtype, which provides information about the prognosis by describing the natural history of a tumor and the therapies that are and are not likely to be successful. The median OS for the 50% of patients with KRAS/NRAS/BRAF WT mCRC is about 30 mo with survival rates of 80% at 1 year, 40% at 3 years, and 20% at 5 years after the start of first-line chemotherapy (Table 2).

SYSTEMIC TREATMENT FOR LIVER METASTASIS CRC

Nearly half of individuals with initial CRC develop liver metastatic diseases from CRC or colorectal liver metastasis (CLM). The resectability of CLM determines how to manage it, and interdisciplinary approaches are frequently used. Conversion treatment (CT), a kind of systemic treatment, is used for liver metastases that are initially incurable. Both the number (4 *vs* > 4) and size (diameter < 6 cm *vs* ≥ 6 cm) of CLMs are independent variables linked to successful CT[40,41].

Targeted treatment

FOLFOX (5-fluorouracil [5-FU], leucovorin, and oxaliplatin) and FOLFIRI (5-FU, leucovorin, and irinotecan) are the mainstays of systemic chemotherapy used to treat mCRC. EGFR inhibitors (EGFRis) for RAS WT tumors (cetuximab [Cet] and panitumumab [Pan]) and anti-vascular endothelial growth factor (VEGF) (bevacizumab [Bev]) are the two main groups of medications now added to these chemotherapy regimens.

Resectable CLM with no extrahepatic metastasis

There is debate regarding the benefits of adding a targeted treatment, however, the addition of Bev or Cet to FOLFOX or FOLFIRI is tolerable in resectable liver metastases. However, a single-arm phase 2 study found that the addition of Bev to the capecitabine and oxaliplatin combination (CAPOX) (six cycles with no Bev on the final cycle), before surgery in high-risk CRC, had a remarkable objective response rate (ORR) of 73%[42]. In 2020, Bridgewater *et al*[43] conducted a multicenter, open-label, randomized, controlled, phase 3 trial to investigate the effects of Cet plus chemotherapy compared with those given chemotherapy alone in 257 adult patients (aged ≥ 18 years) with KRAS WT (codons 12, 13, and 61) resectable or suboptimally resectable. At a median follow-up of 66 mo after the last patient was recruited, this analysis was conducted. In the chemotherapy alone group, the median PFS was 22.2 mo, whereas in the chemotherapy plus Cet group, it was 15.5 mo (P = 0.304). In the chemotherapy alone group, the median OS was 81.0 mo, but in the chemotherapy plus Cet group, it was 55.4 mo (P = 0.036). The status of pathological resection or the preoperative response were secondary outcomes that did not significantly differ between groups. High-risk CRC in this study included those with synchronous liver metastases, metastatic disease discovered within a year of initial resection, primary tumors with positive lymph nodes, CLMs > 1 or > 5 cm, and positive carcinoembryonic antigen (CEA) levels. They concluded that Cet had no effect when used with perioperative treatment. As a result, targeted therapy is deemed ineffective in CRC patients with resectable CLM, whereas Bev may be beneficial in high-risk patients. FOLFOX adjuvant treatment is advised following resection.

Unresectable CLM with potential for resection and no extrahepatic metastasis

In high-risk CRC (> 4 metastases, diameter > 5 cm, poor viable liver function if undertaking upfront resection, or inability to maintain liver vascular supply), neoadjuvant treatment (NAT) with CAPOX plus Bev achieved a 78% ORR[44]. Of the 46 patients included, 40% of the individuals with unresectable disease upon diagnosis got a resection. Four patients responded so well that they were kept under surveillance without having surgery. In comparison to FOLFOX, Bev with FOLFOXIRI (combination of 5-FU/leucovorin (LV), oxaliplatin, and irinotecan) had a greater resection rate (61% *vs* 49%), R0 resection rate (49% *vs* 29%), ORR (81% *vs* 62%), and mean PFS (18.6 m *vs* 11.5 m)[45]. After a hepatic artery infusion chemotherapy pump has been installed, adding Bev to the chemotherapy has no survival benefit. On the other hand, it worsens liver toxicity (hyperbilirubinemia > 3 mg/dL) and is not advised[46]. In the POCHER study, 60% (25/43) of the 43% of patients with unresectable CLM who

Table 2 Chemotherapy and targeted therapies for metastatic colorectal cancer patients

Ref.	Country	Drug(s)	Number of patients	Study phase	ORR, %	Mean OS in mo	Mean PFS in mo	Results
Resectable CLM with no extra-hepatic metastasis								
Gruenberger *et al*[42]	Austria	Capecitabine, oxaliplatin plus bevacizumab	56	2	73.2	-	-	Bevacizumab can be safely administered until 5 wk before liver resection in patients with metastatic CRC without increasing the rate of surgical or wound healing complications or the severity of bleeding
Bridgewater *et al*[43]	United Kingdom	Oxaliplatin, L-folinic acid, fluorouracil or capecitabine, oxaliplatin plus cetuximab	257	3	-	81.0/55.4 (Chemo/Chemo+)	22.2/15.5 (Chemo/Chemo+)	In the perioperative setting, patients with operable diseases are at a disadvantage in terms of OS; hence, cetuximab should not be used in this setting
Unresectable CLM with potential for resection and no extra-hepatic metastasis								
Wong *et al* [44]	United Kingdom	Capecitabine, oxaliplatin plus bevacizumab	46	-	78.0	-	-	A high response rate for patients with CLMs with poor-risk features not selected for upfront resection and converted 40% of patients to resectability
Gruenberger *et al*[45]	United Kingdom, Austria, France, and Spain	Bevacizumab plus FOLFOX-6 (5-fluorouracil folinic acid oxaliplatin) or FOLFOXIRI (5-fluorouracil folinic acid oxaliplatin irinotecan)	80	2	81/62 (BF/BF6)	-	18.6/11.5 (BF/BF6)	In patients with CLM that were originally unresectable, bevacizumab-FOLFOXIRI was correlated with better response and resection rates as well as a longer PFS than bevacizumab-mFOLFOX-6
Garufi *et al* [47]	Italy	Cetuximab plus chrono-IFLO (chrono-irinotecan 5-fluorouracil leucovorin oxaliplatin)	43	2	-	37	-	Cetuximab in combination with chrono-IFLO resulted in 60% full resectability of CLM patients
Folprecht *et al*[48]	Germany	Cetuximab plus FOLFOX (5-fluorouracil folinic acid oxaliplatin) or FOLFOXIRI (5-fluorouracil folinic acid oxaliplatin irinotecan)	56/55 (CFX/CFI)	-	-	35.7/29.0 (CFX/CFI)	10.8/10.5 (CFX/CFI)	Both FOLFOX/FOLFIRI with cetuximab appear to be effective conversion therapy regimens in patients with KRAS codon 12/13/61 wild-type tumors. Thus, liver surgery can be deemed curative or as a second line of therapy in people who are not cured
Unresectable CLM								
Rivera *et al* [50]	Spain, Germany, United States, Belgium, Switzerland, and Italy	mFOLFOX6 plus panitumumab or bevacizumab	170/156 (RAS WT/RAS WT/BRAF WT)	-	RAS WT 65/60, RAS WT/BRAF WT 65/62 (FXP/FXB)	RAS WT 36.9/28.9, RAS WT/BRAF WT 46.3/28.9 (FXP/FXB)	RAS WT 12.8/10.1, RAS WT/BRAF WT 13.1/10.1 (FXP/FXB)	Panitumumab in combination with mFOLFOX6 is a successful first-line therapy for individuals with RAS WT and RAS

								WT/BRAF WT mCRC
Stintzing *et al*[53]	Germany	FOLFIRI (5-fluorouracil leucovorin irinotecan) plus cetuximab or bevacizumab	400	3	65.3/58.7 (FIC/FIB)	33.1/25.0 (FIC/FIB)	10.3/10.2 (FIC/FIB)	In the first-line therapy of patients with RAS wild-type metastatic colorectal cancer, FOLFIRI with cetuximab may be preferable than FOLFIRI plus bevacizumab
Pfeiffer *et al* [58]	Denmark	Trifluridine plus tipiracil hydrochloride (TAS-102) or TAS-102 plus bevacizumab	47/46 (T/TB)	2	51/67 (T/TB)	6.7/9.4 (T/TB)	2.6/4.6 (T/TB)	In terms of efficacy and safety, bevacizumab may be an effective companion for TAS-102 in patients with chemorefractory metastatic colorectal cancer
Cremolini *et al*[59]	Italy	Cetuximab plus irinotecan	13/12 (RWT/RMT)	2	-	12.5/5.2 (RWT/RMT)	4.0/1.9 (RWT/RMT)	In patients with RAS and BRAF wild-type mCRC who have acquired resistance to first-line irinotecan and cetuximab-based treatment, a re-challenge approach with cetuximab and irinotecan may be effective. The examination of RAS mutational status on cDNA may aid in the selection of potential patients
Sartore-Bianchi *et al* [60]	Italy	Panitumumab (re-challenge)	25	-	30	13.7	4	Interventional liquid biopsies can be used efficiently and safely to guide anti-EGFR re-challenge treatment with panitumumab in patients with mCRC
Kopetz *et al* [63]	United States	Irinotecan cetuximab or irinotecan cetuximab plus vemurafenib	50/50 (IC/ICV)	-	-	12/12 (IC/ICV)	2.0/4.2 (IC/ICV)	Vemurafenib in combination with cetuximab and irinotecan is an active combination that increases PFS. This is a well-planned research based on a solid grasp of the mechanisms of adaptive resistance in mCRC
Tabernero *et al*[64]	United States, France, Italy, Spain, United Kingdom, Germany, Australia, Hong Kong, Norway, Switzerland, Japan, South Korea, and the Netherlands	Encorafenib cetuximab binimetinib or encorafenib cetuximab or FOLFIRI cetuximab	224/220/221 (3D/2D/CD)	3	26.8/19.5/1.8 (3D/2D/CD)	9.3/9.3/5.9 (3D/2D/CD)	-	When compared to conventional chemotherapy, encorafenib plus cetuximab improved OS, ORR, and progression-free survival in previously treated patients with metastatic disease. Encorafenib with cetuximab is a new standard-of-care regimen for previously treated patients with BRAF V600E mCRC, according to main and revised studies

Siena *et al* [68]	United States, Italy, United Kingdom, Spain, and Japan	Trastuzumab deruxtecan (DS-8201)	53/7/18 (A/B/C)	2	45.3 (A)	5.4 (A)	6.9 (A)	Trastuzumab deruxtecan shown promising and sustained effect in HER2-positive mCRC that was resistant to conventional therapy, as well as a favorable safety profile
Mayer *et al* [71]	Belgium, Italy, Japan, Spain, Australia, and United States	Trifluridine plus tipiracil hydrochloride (TAS-102) or placebo	800 (2:1)	-	1.6/0.4 (TAS/placebo)	7.1/5.3 (TAS/placebo)	2.0/1.7 (TAS/placebo)	A significant number of Japanese and Western patients with mCRC who had had a lot of prior treatment, including those whose condition was resistant to fluorouracil, were shown to respond clinically to TAS-102

2D: Encorafenib plus cetuximab; 3D: Encorafenib plus cetuximab plus binimetinib; A: Cohort A; B: Cohort B; BF: Bevacizumab plus FOLFOXIRI; BF6: Bevacizumab plus FOLFOX-6; BRAF: Protein kinase B-Raf; C: Cohort C; CD: FOLFIRI plus cetuximab; CFI: Cetuximab plus FOLFOXIRI; CFX: Cetuximab plus FOLFOX; CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor; FIB: FOLFIRI plus bevacizumab; FIC: FOLFIRI plus cetuximab; FOLFIRI: Folinic acid, fluorouracil, and irinotecan hydrochloride; FXB: mFOLFOX6 plus bevacizumab; FXP: mFOLFOX6 plus panitumumab; HER2: Human epidermal growth factor receptor 2; IC: Irinotecan plus cetuximab; ICV: Irinotecan plus cetuximab plus vemurafenib; mCRC: Metastatic colorectal cancer; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival; RAS: Rat sarcoma virus; RMT: Rat sarcoma virus mutate-type; RWT: Rat sarcoma virus wild-type; T: Trifluridine plus tipiracil hydrochloride; TB: Trifluridine plus tipiracil hydrochloride plus bevacizumab; WT: Wild-type.

received chronomodulated irinotecan (Iri) and 5-FU/LV on days 2-6 every 2 wk as NAT experienced full resections[47]. Twenty-nine participants with more than four lesions and nine patients with more than 5 cm in diameter made up the study population. The 2-year survival rate was 68% for the whole population. In people who have had surgery, it may reach 80%. As a result, research was conducted to evaluate the role of EGFRi in NAT or POT. When Cet was given to FOLFOX or FOLFIRI in patients with unresectable CLM at diagnosis who were eligible (with KRAS WT codons 12, 13, and 61), it improved long-term survival[48].

Unresectable CLM

First-line plus chemotherapy: Bev is advised as first-line treatment for RAS-mutated tumors in mCRC with CLM and without surgical consideration. A meta-analysis of two randomized control trials and three observational studies revealed that Cet had a higher OS, ORR, and complete response rate than Bev in RAS-WT malignancies (with chemotherapy as the backbone). The same trial found no appreciable differences between the two medications in PFS, disease control rate, or partial response rate[49]. In the PEAK study, Pan outperformed Bev in terms of PFS while maintaining the same OS[50]. In the past 5 years, a lot of research has been done on the function of the main tumor side (right *vs* left). Regardless of the CLM status, two meta-analyses have conclusively demonstrated that left-sided cancers react better to EGFRi than Bev[51,52]. However, there was no statistically significant difference in OS or PFS between Bev and EGFRi in tumors on the right side. In the PEAK study and the FIRE-3 trial, EGFRi with chemotherapy produced deeper responses in RAS-WT tumors than Bev plus chemotherapy. Tolerability is a crucial consideration when choosing a medication. Patients with a history of thromboembolic illness, uncontrolled hypertension, proteinuria, significant bleeding risk, or gastrointestinal perforation are not advised to get anti-VEGF medication. As a result, EGFRi (Cet or Pan) is preferable over Bev plus chemotherapy in left-sided tumors in RAS-WT mCRC with CLM, although either of them can be administered in right-sided tumors[53,54]. There is not any conclusive proof that Pan or Cet is superior to the other. Patients who experience adverse reactions to Cet, a mouse-based monoclonal antibody (mab), frequently prefer Pan since it is a humanized mab.

Second-line plus chemotherapy: The continuation of Bev in the second line after first-line treatment improved PFS and OS without a worsening in side events. There is insufficient information to definitively conclude that changing eligible patients from Bev to Cet or Pan following clinical progression would be beneficial[55,56]. There is no advantage to switching Bev to another VEGF medication. Patients who progress on oxaliplatin-based therapy (FOLIRI-naive) benefit from ziv-aflibercept or ramucirumab when used in conjunction with FOLIRI[57]. Bev may also be used in the third or fourth line of TAS-102 (trifluridine and tipiracil hydrochloride)[58]. Continuing EGFRi has no advantage for OS if Cet or Pan are used as the first-line treatments[59]. Circulating tumor DNA can be used to identify acquired resistance, which may manifest in a small number of patients. It is anticipated that this resistance would fade with time. As a result, switching to Bev is advised[60,61]. After the

disease progressed, switching from Cet to Pan or vice versa is not useful. If not used in the first-line setting, EGFRi may be administered either alone (monotherapy) or with Iri, FOLFIRI, or FOLFOX but not with CAPEOX, or to patients who cannot handle chemotherapy[62]. In ongoing studies, they are being used with additional medications such as immune checkpoint inhibitor (ICI), BRAF, and tyrosine kinase inhibitors.

Patients with resistant mCRC who have *BRAF* mutations, notably BRAF V600E and KRAS WT, may benefit from a combination therapy that combines BRAF inhibitors with Cet or Pan[63]. In the interim analysis of the BEACON trial, triple treatment with the MEK inhibitor (binimetinib) demonstrated a survival benefit over the control group (Cet plus Iri or FOLFIRI) and combination therapy. A more recent investigation, however, found no benefit of triplet treatment over doublet treatment compared to the control group[64]. In the initial studies with Pan, other BRAF inhibitors, dabrafenib and vemurafenib, with/without MEK inhibitors (trametinib), showed favorable outcomes[65]. In approximately 6% of CRCs, HER2 is amplified or overexpressed[66]. Trastuzumab, a HER2 inhibitor, in combination with pertuzumab (an mAb that prevents dimerization of HER2 and HER3) or lapatinib (inhibitor that binds to the cytoplasmic ATP-binding site inhibitor EGFR/HER1 and HER2 receptors), is well tolerated in refractory mCRC patients with HER2 amplification and RAS/BRAF WT[67]. HER2-amplified, a humanized anti-HER2 Ab combined with a topoisomerase I inhibitor, known as trastuzumab deruxtecan (T-dx), demonstrated an excellent ORR (45.3%) after a median follow-up of 27.1 wk in refractory mCRC[68]. Patients who were resistant to HER2 inhibitors also showed activity when given T-dx. Neurotropic tyrosine receptor kinase (*NTRK*) genes code for the tyrosine kinase receptors that control cell growth, and rearrangements result in unregulated cell proliferation. It was first discovered in CRC and occurs in just 0.3% of solid tumors[69]. There was 7% CRC in the studies that administered NTRK inhibitors to solid tumors, such as entrectanib and larotectinib. Patients with *NTRK* mutations may choose this as a treatment option[70].

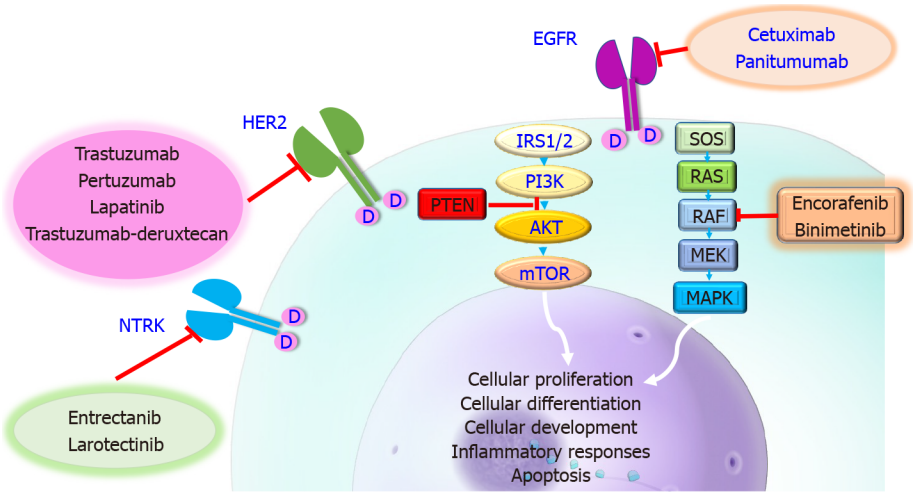
A multinational, randomized, placebo-controlled, phase 3 trial of a multikinase tyrosine kinase inhibitor is called the CORRECT trial. The regorafenib therapy group outlived the placebo group by 1.4 years (mOS for regorafenib is 6.4 years *vs* 5 years for placebo; $P = 0.005$). The use of a regulator was associated with higher medication toxicity (93% in the regorafenib *vs* 61% in the placebo). Hand-foot skin reactions were the most common adverse event (AE) (83%) observed, followed by tiredness (48%) and hypertension (36%). ICIs including ipilimumab/nivolumab, pembrolizumab, and EGFRi are now being explored in conjunction with it. Its usage is frequently contrasted with that of the TAS-102, which had an OS improvement of 1.8 m above placebo. Combinations of ICI and EGFRi are being investigated [71] (Figure 2).

IMMUNOTHERAPY

The goal of immunotherapy is to use the immune system to fight cancer. For patients with mCRC that is mismatch-repair-deficient (dMMR) or MSI-H (dMMR/MSI-H mCRC), ICIs have emerged as a very effective treatment. ICIs modify the interaction of T cells, antigen-presenting cells, and tumor cells to help unleash suppressed immune responses. The FDA approved pembrolizumab and nivolumab (with or without Ipilimumab) for the treatment of these patients due to their effective, stable, and long-lasting responses. The fundamental difficulty is to offer the advantage of immunotherapy to the great majority of mCRC patients who are mismatch-repair-proficient (pMMR), microsatellite-stable (MSS), or have low MSI (MSI-L), since mCRC is characterized by an inadequate number of mutant tumor antigens[72].

ICIs-based immunotherapy

Use of ICIs in DMMR/MSS mCRC: To preserve DNA integrity, MMR is essential. CRC can be classified as dMMR or pMMR CRC based on the detection of the MMR proteins MLH1, MSH2, MSH6, or PMS2 utilizing immunohistochemical staining. Moreover, insertions and deletions can cause MSI, which can be precisely identified by PCR or next-generation sequencing, resulting in a change in microsatellite length. Major histocompatibility complex (MHC) class I peptide complexes, including mutant peptides that might be identified as neoantigens and subsequently increase immune cell priming and infiltration, are present on the surface of tumor cells in dMMR-MSI-H malignancies. T helper 1 CD4+ T cells, macrophages, and CD8+ tumor-infiltrating lymphocytes (TILs) enter the TME and produce interferon gamma. Programmed cell death ligand-1, cluster of differentiation 80 (CD80), and CD86 of the B7 family are examples of T cell inhibitory ligands that dMMR-MSI-H tumor cells persistently upregulate to support immune escape[73-75]. The percentage of dMMR-MSI-H CRCs, which accounts for about 15% of all CRCs, is correlated with tumor stage. dMMR-MSI-H cancers make up about 5%-20% of stage 2 and 11% of stage 3, but only 5% of stage 4. Additionally, dMMR-MSI-H is a predictive biomarker for individuals at various phases of their condition. Patients with dMMR-MSI-H tumors have a much better prognosis than those with pMMR-MSI-L cancers in stages 2 and 3. Surprisingly, individuals with stage 4 dMMR-MSI-H have a poor prognosis yet respond well to immune checkpoint inhibition[76,77].



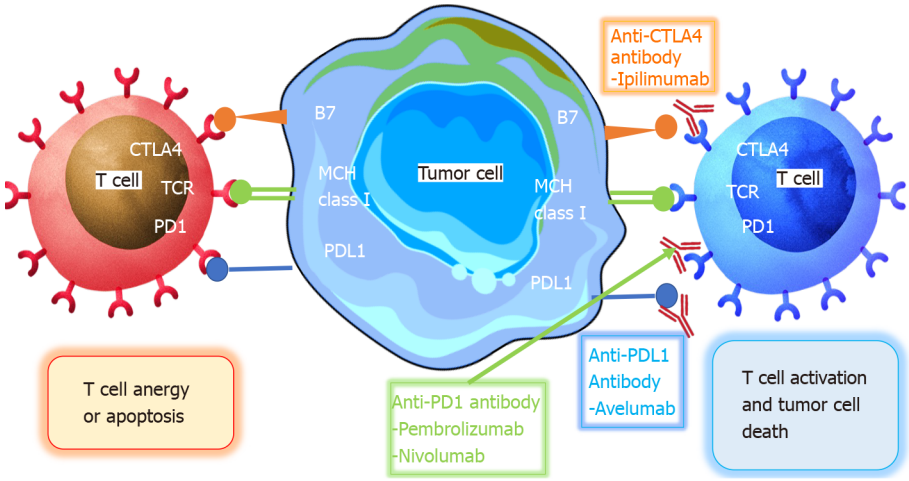
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Figure 2 Targeted therapy for metastatic colorectal cancer. AKT: Ak strain transforming; D: Down regulation pathway; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2; IRS1/2: Insulin receptor substrates 1 or 2; MAPK: Mitogen activated protein kinase; mTOR: Mammalian target of rapamycin; MEK: Mitogen-activated protein kinase kinase; NTRK: Neurotrophic tyrosine receptor kinase; PTEN: Phosphatase and tensin homolog; PI3K: Phosphoinositide 3-kinases; RAF: Rapidly accelerated fibrosarcoma; RAS: Rat sarcoma virus; SOS: Son of sevenless.

Le *et al*[78] conducted a phase 2 trial in 41 patients with progressive mCRC with or without dMMR to investigate the clinical efficacy of pembrolizumab, an anti- Programmed cell death protein 1 (PD-1) ICI, in 2015. Pembrolizumab was given intravenously every 14 d at a dosage of 10 mg/kg body weight to patients with dMMR CRC, pMMR, and patients with dMMR who were not colorectal. The immune-related ORR and PFS for dMMR CRC were 40% and 78%, respectively, and 0% and 11% for pMMR CRC. In the group with dMMR CRC, the median PFS and OS were not attained, but in the cohort with pMMR CRC, they were 2.2 and 5.0 mo, respectively (hazard ratio [HR] for PFS and death = 0.10 and 0.22). Responses in patients with dMMR non-CRC were comparable to those in individuals with dMMR CRC. High somatic mutation loads were related to longer PFS (P = 0.02), and whole-exome sequencing found that dMMR tumors had an average of 1782 somatic mutations per tumor, compared to 73 somatic mutations in pMMR tumors (P = 0.007). They concluded that MMR status predicted the therapeutic benefit of immune checkpoint inhibition with pembrolizumab (Figure 3).

In 2020, the KEYNOTE-164 study analyzed pembrolizumab's effectiveness in 124 patients with dMMR/MSI-H mCRC who had undergone treatment. The ORR was 32% among the 63 patients examined, and the median PFS was 4.1 mo. The overall median survival rate has not yet been reached. The percentages of OS and PFS at 1 year were 41% and 76%, respectively. A single ICI therapy for individuals with dMMR/MSI-H mCRC demonstrated sustained anticancer efficacy[79]. In a phase 3, open-label study, 307 mCRC patients with dMMR/MSI-H who had not previously received treatment were enrolled to assess the effectiveness of PD-1 blockers or chemotherapy as first-line treatments. They were given a 1:1 random assignment to undergo chemotherapy (5-FU-based treatment with or without bevacizumab or cetuximab) every 2 wk, or pembrolizumab at a dosage of 200 mg every 3 wk. After advancement of the condition, patients taking chemotherapy could switch to pembrolizumab therapy. Of the 307 patients enrolled, 153 received single-agent pembrolizumab and 154 received chemotherapy. The median PFS time was 16.5 mo in the pembrolizumab group and 8.2 mo in the chemotherapy group at a median follow-up of 32.4 mo (HR = 0.60; P < 0.001). Significant differences were seen between the pembrolizumab group's 12- and 24-mo PFS values, which were 55% and 48%, respectively, *vs* 37% and 19% in the chemotherapy group. These data show that, compared to chemotherapy, pembrolizumab demonstrates more stable anticancer activity and fewer treatment-related AEs[80]. Based on the compelling evidence from this trial, the FDA approved pembrolizumab for the first-line treatment of patients with dMMR/MSI-H or advanced, unresectable, or metastatic CRC in June 2020.

In certain clinical studies, the use of nivolumab, another PD-1 inhibitor that targets dMMR/MSIH CRC, is also being investigated. Nivolumab's effectiveness was examined in the phase 2 study CheckMate 142 on 74 patients with dMMR/MSI-H mCRC. The ORR was 31%, and 69% of patients had disease control for 12 wk or longer. The median duration of response was not attained. The PFS and OS rates during the past 12 mo were 50% and 73%, respectively. Nivolumab's safety profile in this cohort study was consistent with that previously reported in other solid tumor trials, and there were no additional AEs noted[81]. Nivolumab was approved by the FDA in August 2017 for the treatment of dMMR/MSI-H mCRC in adults and children older than 12-years-old on the basis of these research findings[82]. Nivolumab's administration in conjunction with the cytotoxic T-lymphocyte-associated protein 4 mAb ipilimumab was also investigated in the CheckMate142 trial. Nivolumab 3 mg/kg in combination with ipilimumab 1 mg/kg was given once every 3 wk (four doses) to a total of 119 patients



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Figure 3 Immune check point inhibitors treatment for metastatic colorectal cancer. CTLA4: Cytotoxic T-lymphocyte-associated protein 4; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand; TCR: T cell receptor.

with dMMR/MSI-H mCRC who had not responded to conventional therapy. Nivolumab 3 mg/kg was then given once every 2 wk. The ORR was 55% at a median follow-up of 13.4 mo. In 80% of patients, the disease was under control for at least 12 wk; however, the median PFS was not met. The PFS rates for the 9th and 12th mo were 76% and 71%, respectively. The 9- and 12-mo OS rates were 87% and 85%, respectively, but the median OS was not met[83]. The FDA expedited the approval of nivolumab in combination with ipilimumab for the treatment of patients with dMMR/MSI-H mCRC in July 2018 based on the findings of this trial. Furthermore, the most recent information from the 2-year follow-up was used to update the study outcomes. The ORR and disease control rates determined by the study were 69% and 84%, respectively, over a median follow-up of 29 mo. The 2-year PFS and OS rates were 74% and 79%, respectively, but the median PFS and OS was not attained[84].

After first-line chemotherapy failed, Kim *et al*[85] conducted a prospective, open-label, multicenter phase 2 trial in 2020 to assess the effectiveness and safety of avelumab in 30 mCRC patients who had dMMR)/MSI-H and 3 mCRC patients who had polymerase-epsilon (POLE) mutations. All of the respondents were dMMR/MSI-H, and the ORR was 24.2%. At a median follow-up time of 16.3 mo, the median PFS and OS for all patients was 3.9 and 13.2 mo, respectively. They concluded that in patients with previously treated mCRC carrying dMMR/MSI-H, avelumab demonstrated anticancer efficacy with controllable toxicity. To assess the effectiveness and safety of cetuximab re-challenge treatment combined with avelumab in 71 MSS, 3 MSI-H, and 3 patients with uncertain microsatellite status with mCRC, Martinelli *et al*[86] conducted a single-arm, multicenter phase 2 study in 2021. The patients were given cetuximab (400 mg/m^2, then 250 mg/m^2 weekly) and avelumab (10 mg/kg every 2 wk) until the disease progressed or the side effects became intolerable. With a median OS of 11.6 mo and a median PFS of 3.6 mo, the trial accomplished its primary aim; 4% of grade 3 AEs were diarrhea, and 14% of them were skin eruptions. There were 48 people with WT illnesses and 19 people with mutations. Those with RAS/BRAF WT cDNA had a median OS of 17.3 mo, opposed to patients with mutations, who had a median OS of 10.4 mo. In contrast to patients with mutant cDNA, those with RAS/BRAF WT had a median PFS of 4.1 mo opposed to 3.0 mo. They concluded that an active, well-tolerated challenge treatment for RAS WT mCRC is cetuximab plus avelumab.

Use of ICIs in PMMR/MSS mCRC: pMMR/MSS CRC, which makes up about 95% of all mCRCs, is referred to as a "cold tumor." Single-agent ICI had no effect on pMMR/MSS CRCs in contrast to inflammatory tumors of dMMR/MSI-H. The results of recent investigations on the use of combination ICIs have raised the prospect of enhancing immunotherapy efficacy in this population. ICIs in conjunction with systemic chemotherapy were proven to dramatically increase tumor treatment response in refractory mCRC treated with conventional chemotherapy and immunotherapy, especially in pMMR/MSS CRCs. Existing data show that immunogenic chemotherapy might improve the efficacy of ICIs by increasing tumor infiltration of CD4+ and CD8+ T cells and interrupting the function of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells[87,88]. To investigate ways to make cold tumors heated to boost sensitivity to immunotherapy, a number of ICI-based combination treatment studies tested ICIs in conjunction with chemotherapy, targeted therapy, ICI therapy, and radiation.

Pembrolizumab combined with modified FOLFOX6 was tested in a single-arm, multicenter phase 1b study by Herting *et al*[89] for the treatment of mCRC. In this study, 87% of the participants had pMMR/MSS mCRC. At a median follow-up of 19.9 mo among the 30 patients, the investigators noted an ORR of 57% and the mean time it took for the responding patients to respond was 37.57 wk. The median PFS

time was 8.8 mo, and the median OS was not reached. Recently, two phase 2 trials, AtezoTRIBE and MAYA, evaluating combinations of ICIs with chemotherapy, revived optimism for the use of immunotherapy in pMMR/MSS mCRC patients, indicating a significant breakthrough and a potential basis for future research in this scenario.

AtezoTRIBE trial: Regardless of microsatellite status, 218 patients with unresectable and chemo-naive mCRC were randomized in a 1:2 ratio to receive FOLFOXIRI. The control group received first-line FOLFOXIRI (intravenous 165 mg/m^2 irinotecan, 85 mg/m^2 oxaliplatin, 200 mg/m^2 leucovorin, and 3200 mg/m^2 fluorouracil as a 48-h infusion) plus bevacizumab (5 mg/kg intravenously), and the atezolizumab group received the same regimen plus atezolizumab (840 mg intravenously) in the AtezoTRIBE phase 2 multicenter, open-label, comparative study. According to the randomized arm, both treatments were given for up to eight cycles, then 5-FU with bevacizumab, with or without atezolizumab, was given until the condition progressed, there were unacceptable side effects, or the patient withdrew their consent. PFS was the main endpoint, with a one-sided alpha error of 0.10 and an 85% power. A median follow-up of 19.9 mo was being used. In the atezolizumab group, the median PFS was 13.1 mo, compared to 11.5 mo in the control group (HR = 0.69; P = 0 012). Neutropenia (42% of 142 patients in the atezolizumab group *vs* 36% of 72 individuals in the control group), diarrhea (15% *vs* 13%), and febrile neutropenia (10% *vs* 10%) were the most common all-cause grade 3-4 AEs. A total of 39 patients (27%) in the atezolizumab group and 19 patients (26%) in the control group suffered serious AEs. Acute myocardial infarction and bronchopulmonary hemorrhage caused two (1%) treatment-related fatalities in the atezolizumab group; none were recorded in the control group. They concluded that first-line FOLFOXIRI plus bevacizumab with atezolizumab added was safe and enhanced PFS in patients with mCRC who had not previously received treatment[90].

MAYA trial: MAYA is a prospective single-arm phase 2 trial that included patients with chemo-resistant mCRC who had centrally confirmed MSS status, O6-methylguanine-methyltransferase (MGMT) silence determined by promoter methylation of the MGMT gene, and total immunohistochemical loss of MGMT protein. Only in situations where disease control is obtained during phase 1 are participants to receive two cycles of temozolomide (TMZ) (phase 1), followed by the addition of nivolumab and low-dose ipilimumab (phase 2). The 8-mo PFS rate in patients included in phase 2 of the trial served as the study's main objective. The MAYA trial's design is supported by an intriguing biological theory. In short, the MGMT gene plays a role in repairing DNA damage brought on by alkylating drugs like TMZ[91]. Sensitivity to TMZ is increased when MGMT is inactivated by hypermethylation of its promoter. As demonstrated in studies evaluating the efficacy of TMZ alone or in combination with other chemotherapeutic agents, such as capecitabine and irinotecan, retrospective data revealed that sensitivity to TMZ was primarily restricted to pMMR/MSS tumors with complete MGMT protein loss detected with immunohistochemistry[92]. After the first disease response, MGMT re-expression, the selection of sub-clones that express MGMT, or a hypermutated condition resulting from acquired mutations in MMR genes that might make mCRC sensitive to ICIs can all lead to secondary resistance to TMZ[93]. In the MAYA study, 204 of 703 assessed patients (29%) were found to be molecularly suitable. Overall, 142 of 703 (19%) patients were enrolled in phase 1, with just 33 (5% of the initial 703 screened patients) progressing to phase 2. The 8-mo PFS rate was 36% after a median follow-up of 23.1 mo. The median PFS and OS were 7.0 and 18.4 mo, respectively, with a 45% response rate. Skin rash (6%), colitis (3%), and hypophysitis (3%), were all immune-related side effects of grade 3-4 severity. There were no unanticipated AEs or treatment-related fatalities recorded. They concluded that TMZ priming followed by a combination of low-dose ipilimumab and nivolumab might result in long-term therapeutic benefit in MSS and MGMT-silenced mCRC. These findings should be considered with caution due to the lack of a control arm testing the effectiveness of TMZ monotherapy, which prevented the investigators from distinguishing the effect of immunotherapy addition *vs* TMZ alone. Given that not all patients who are initially susceptible to TMZ develop a hypermutated phenotype, only a subset of individuals may benefit from immunotherapy. Future analyses separating the ORR observed during phase 1 (with TMZ) from the ORR reported during phase 2 (with TMZ plus ipilimumab plus nivolumab), as well as current translational investigations, might reduce this issue.

Despite the need for more mature follow-up data, the good findings from the AtezoTRIBE and MAYA trials bring an end to a long period of stagnation and dismal outcomes in the landscape of immunotherapy in pMMR/MSS mCRC. In the first-line scenario, chemotherapy escalation and TMZ delivery in MGMT-silenced chemo-refractory patients are capable of sensitizing immune-deficient or cold mCRCs to immunotherapy, perhaps rewiring an inflamed/hot TME, then unleashed against the tumor by ICIs. Larger confirmatory and translational trials, however, are required to identify people who benefited the most from these therapies[94].

ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT), a crucial component of tumor immunotherapy, entails the introduction of immunologically active cells that have been grown and altered *in vitro* to have direct anticancer action

against the cancer-stricken host. Chimeric antigen receptor T (CAR-T) cells, TILs, and T cell receptor-engineered T cells are the three ACT types currently being researched for the treatment of cancer. CAR-T cell therapy entails modifying T cells *in vitro* in an MHC-independent manner so that they can target tumor antigens and produce an anticancer immune response. With its tremendous effectiveness in treating leukemia, multiple myeloma, some forms of lymphoma, and mCRC, CAR-T cell therapy has a lot of room to grow[95]. Numerous clinical investigations on the safety and efficacy of CAR-T cell treatment are now being conducted in order to determine its therapeutic potential in the field of CRC. One of the first human trials using CAR-T cells to treat metastatic CRC was published by Hege *et al*[96]. It consisted of two phase 1 experiments with the same CART72 cells (C9701 and C9702). As first-generation CAR-T cells with a CD3-zeta intracellular signaling domain that specifically targeted the tumor-associated glycoprotein-72, CART72 cells were created. The way CART72 was administered in the two studies was different. In trial C9702, patients with CLM received direct hepatic artery infusions, whereas in trial C9701, CART72 was administered intravenously in increasing dosages. Despite a brief blood persistence and modest trafficking to tumor tissue, the data indicated a good safety profile. In addition, rapid clearance following CAR-T cell infusion was linked to CART72 immunogenicity.

Guanylylcyclase2C (GUCY2C) was mentioned by Magee *et al*[97,98] as a potential CAR-T cell target. In a mouse model lacking autoimmunity, they demonstrated that GUCY2C CAR-T cells may cure parenchymal CRC metastases. Additionally, they showed that GUCY2C targeted CAR-T cell treatment works well against metastatic cancers in mouse models and in human CRC xenograft models. Zhang *et al*[99] conducted a phase 1 study using CEA-positive CRC patients to create and assess CEA CAR-T cell treatment in 10 resistant and relapsed CRC patients with metastases. CAR-T cells were administered at five increasing dosage levels (1×10^5 to 1×10^8/CAR+/kg cells) to these individuals. The findings demonstrated that there were no significant side effects of CAR-T treatment. Seven of the ten patients—those with progressing illness throughout prior therapies—had stable disease following CAR-T cells therapy. Two patients had tumors removed, and two others had stable illnesses for more than 30 wk. They concluded that CEA CAR-T cell treatment, even at large dosages, was well tolerated in CEA+ CRC patients and that the majority of the treated patients showed some effectiveness.

Several ongoing trials are investigating the use of CAR-T cell in the treatment of CRC[100]. These included the safety, cellular kinetics, and efficacy of CYAD-101, an allogenic CAR-T cell therapy targeting ligands of NKG2D that was administered concurrently with FOLFOX, the efficiency and safety of NKR-2 CAR-T cells, EGFR and EGFR IL 12 CAR-T cell safety and feasibility, the use of anti-carcinoembryonic antigen targeted CAR-T cells, and investigating the efficacy and safety of HER2 chimeric antigen receptor-modified adenovirus-specific cytotoxic T lymphocytes administered in association with intratumoral injection of CAdVEC in patients with unresectable mCRC.

CONCLUSION

With the discovery and comprehension of several molecular and anatomical indicators, the landscape of systemic therapy for mCRC has significantly changed. To find the most effective treatment solution for mCRC patients, a baseline, thorough molecular study is now required. The effect of RAS, BRAF, HER2, POLE, MMR, MSS, and MSI status on therapy choice is summarized in this article. The selection of an efficient first-line therapy, which should consider both clinical factors and molecular signs, is crucial for deciding treatment outcomes. The second-line regimen is chosen based on the systemic therapies utilized in the first. For patients with RAS WT, third-line treatment, which includes EGFR inhibitors, should consider molecular profiling. Immunotherapy with pembrolizumab or nivolumab plus ipilimumab may be an option for patients with high microsatellite instability diseases.

The TME has been identified as a crucial player in CRC tumor growth and metastasis. This process involves all of the components from both bacteria and the host. Although each component has a unique function in CRC growth and metastasis, the majority of them act as a double-edged sword, promoting or inhibiting tumor expansion depending on the setting. TME-modulating treatment methods are showing promise. Many researches have confirmed that altering the TME can result in greater anti-tumor actions. Clinical investigations have indicated that TME remodeling has a high potential for improving medication therapeutic efficacy. Furthermore, because tumors tend to acquire resistance, monotherapy is frequently insufficient. Combining TME remodeling techniques with other potential therapies, such as targeted treatment and immunotherapy, is another component we need to investigate in the near future to reduce treatment resistance.

FOOTNOTES

Author contributions: Leowattana W wrote the paper; Leowattana T and Leowattana P collected the data.

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MINIREVIEWS

Gastrointestinal microbiome and cholelithiasis: Current status and perspectives

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Abstract

Cholelithiasis is a common digestive disease affecting 10% to 15% of adults. It imposes significant global health and financial burdens. However, the pathogenesis of cholelithiasis involves several factors and is incompletely elucidated. In addition to genetic predisposition and hepatic hypersecretion, the pathogenesis of cholelithiasis might involve the gastrointestinal (GI) microbiome, consisting of microorganisms and their metabolites. High-throughput sequencing studies have elucidated the role of bile, gallstones, and the fecal microbiome in cholelithiasis, associating microbiota dysbiosis with gallstone formation. The GI microbiome may drive cholelithogenesis by regulating bile acid metabolism and related signaling pathways. This review examines the literature implicating the GI microbiome in cholelithiasis, specifically gallbladder stones, choledocholithiasis, and asymptomatic gallstones. We also discuss alterations of the GI microbiome and its influence on cholelithogenesis.

Key Words: Gallstone; Cholesterol gallstone; Common bile duct stone; Bile acid; Bile microbiome; Gastrointestinal microbiome

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Core Tip: Cholelithiasis is a common digestive disease that imposes significant global health and financial burdens. High-throughput screening demonstrated the relationship between bile, gallstones, and the fecal microbiome in cholelithiasis and provided evidence that gastrointestinal (GI) microbiota dysbiosis is associated with gallstone formation. We summarize the current literature, pool the available cholelithiasis-related studies about the GI microbiome, discuss the underlying mechanisms by which the GI microbiome modulates cholelithiasis, and suggest potential microbiome-targeting therapeutics for cholelithiasis prevention.

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INTRODUCTION

Cholelithiasis or gallstones is a common digestive disease with a high incidence and relatively low mortality. With an overall prevalence of 11.0% in China[1], cholelithiasis is an important public health problem. Gallstone disease's prevalence ranges from 0.60 to 1.39% per year in European population surveys[2]. In the United States, 20 to 25 million adults have cholelithiasis, costing more than $6 billion annually[3,4]. Although many cholelithiasis patients are asymptomatic, approximately one-third develop biliary-pancreatic diseases, including acute or chronic cholecystitis, cholangitis, pancreatitis, and even biliopancreatic cancerous lesions. These diseases impose significant global health and financial burdens.

The study of the human microbiome, particularly the gastrointestinal (GI) microbiome, has rapidly evolved in recent decades, primarily due to the new generation of sequencing technology. The composition, diversity, and richness of microbial communities in the GI tract change during disease states. In addition to definite intestinal dysbiosis-related digestive diseases, the GI microbiome is altered in many biliary disorders, which are rarely traditionally considered microbial in etiology. A predictive model including the genera *Burkholderia*, *Caballeronia*, and *Paraburkholderia* was better able to predict cholangiocarcinoma than the tumor marker carbohydrate antigen 19-9[5]. The proportion of *Streptococcus* is proportionate to the severity of primary sclerosing cholangitis[6].

Investigations of the GI microbiome have extended to cholelithiasis. In the early 20th century, studies supported the existence of interactions between gallstones and bacteria such as *Helicobacter*. Although it has been recognized that specific bacteria contribute to gallstone formation, studies have recently shown that a complex GI microbiome rich in *Desulfovibrionales* promotes gallstone formation by regulating bile metabolism. This review examines the literature implicating the role of the GI microbiome in cholelithiasis, including the biliary, gallstone, and fecal microbiomes (Table 1). We will summarize the literature and discuss mechanisms by which the GI microbiome modulates cholelithiasis; finally, we discuss advances in microbiome-based therapeutics.

THE GI MICROBIOME RELATED TO CHOLELITHIASIS

The biliary microbiome

Studies suggest that the healthy biliary tract is a sterile environment. With the development of sequencing technology, studies revealed the existence of a biliary microbiome. Stewart *et al*[7] detected bacteria in bile samples in nearly a third of gallstone patients. The first study to identify the existence of human biliary microbiota in the gallbladder described the composition of human biliary microbiota using 16S ribosomal RNA (rRNA) gene sequencing. The healthy biliary microbiota is dominated by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* at the phylum level[8]. In addition to bacteria, *Cyanobacteria* and *Spirochaetes* were also present in low amounts.

There are some resemblances and dissimilarities between the biliary and GI microbiomes. The comparative metagenomic analysis demonstrated no significant differences between the GI tract and bile in the predominant phylum *Firmicutes* and the rare phylum *Fusobacteria*. However, the microbial diversity in the biliary tract is more diverse than in the GI tract[9]. The bile duct and duodenum share the core microbiota with the genus *Escherichia–Shigella*, *Fusobacterium*, and *Enterococcus*[10]. Given that the bile duct is anatomically connected to the GI tract *via* the duodenal papilla, it was hypothesized that the biliary microbiota originates from intestinal bacteria and migrates retrograde into the biliary tract. Consistent with the hypothesis, studies demonstrated that the biliary microbiota shared a compositional similarity to duodenal microbiota, and all bacteria in bile were detected in the upper GI using 16S

Table 1 Studies of the gastrointestinal microbiome in cholelithiasis

Microbial changes	Samples	Disease types *vs* control	Methods	Ref.
↑*Desulfovibrionales*	Feces	Cholesterol gallstone *vs* gallstone-free	16S sequencing	Hu *et al*[51]
↑*Megamonas*, *Comamonas*, *Ruminococcaceae*_UCG-014, *Coprobacillus*, *Adlercreutzia*, unclassified_*p*_*Firmicutes*, *Morganella*, CHKCI002, and *Tyzzerella*_4; ↓*Ruminococcaceae*_UCG-008, *Sutterella*, GCA-900066755, *Butyricicoccus*, unclassified_*o*_*Lactobacillales*, and *Lachnospiraceae*_ND3007_group	Feces	Asymptomatic gallstone *vs* gallstone-free	16S sequencing	Song *et al*[33]
↓*Akkermansia muciniphila*, *Prevotella spp.*, *Bifidobacterium adolescentis*, *Alistipes spp.*, *Bacteroides spp.*, *Dorea spp.*, *Methanobacteria*, *Methanobrevibacter smithii*, *Ruminococcus spp.*, and *Faecalibacterium prausnitzii*	Feces	Cholesterol gallstone *vs* pigment gallstone	16S sequencing	Georgescu *et al*[37]
↑7α-dehydroxylating bacteria	Feces	Gallstone *vs* gallstone-free	Culture	Wells *et al*[49]
↑*Proteobacteria*; ↓*Faecalibacterium*, *Lachnospira*, and *Roseburia*	Feces	Gallstone *vs* controls	16S sequencing	Wu *et al*[9]
↑*Aeromonas*, *Enterococcus*, Unclassified_*Enterobacteriaceae*, and *Citrobacter*↓*Prevotella*, *Alloprevotella*, *Nesterenkonia*, and *Pyramidobacter*	Bile	Recurrent CBD stone *vs* new-onset CBD stone	16S sequencing	Chen *et al*[19]
↑*Synergistetes*; ↓*Bacteroidetes*, and *Actinobacteria*	Bile	Recurrent CBD stone *vs* primary CBD stone	16S sequencing	Tan *et al*[20]
↑*Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Veillonellaceae*	Bile	Gallstone *vs* controls	16S sequencing	Molinero *et al*[8]
↑*Brevundimonas* and *Prevotella*_1; ↓*Actinomyces*, *Proteus*, *Clostridiumsensu_stricto*, *Klebsiella*, *Actinobacillus*, *Lachnospiraceae*_UCG-008, *Butyrivibrio*, *Roseburia*, *Porphyromonas*, *Streptococcus*, *Helicobacter*, *Enterobacter*	Bile	Primary CBD stone *vs* controls	16S sequencing	Lyu *et al*[12]
↑*Alcaligenaceae*	Bile	Primary bile duct stone *vs* secondary bile duct stone	16S sequencing	Feng *et al*[18]

CBD: Common bile duct.

sequencing[11,12]. It is noteworthy that the intestinal microbiome contributes to the heterogeneity of the biliary microbiome in cholelithiasis patients, despite the high prevalence of oral cavity and respiratory tract inhabitants to intestinal inhabitants[13].

Because of interactions between the biliary microbiome and metabolic disease, investigators analyzed the bacterial composition associated with cholelithiasis. A Colombian study demonstrated a predominance of *Pseudomonas spp*. in gallbladder tissue and bile[14]. To compare the difference in bile microbiome between gallbladder stone patients and healthy individuals, Molinero *et al*[8] measured bile samples using 16S rRNA sequencing. They demonstrated that the relative abundance of the family *Propionibacteriaceae* in patients with gallbladder stones was lower than in healthy controls. In contrast, the relative abundance of the family *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Veillonellaceae* was higher.

Short-chain fatty acids (SCFAs) consist of butyrate, propionate, and acetate; these are associated with inflammatory diseases and cancers and may serve as nutritional and therapeutic agents in diseases[15,16]. Evidence suggests that low levels of expression of bacteria-producing SCFAs harm the microbial balance of the biliary tract, affecting gallstone formation. There is a significantly decreased abundance of *Clostridiumsensu_stricto*, *Lachnospiraceae* _UCG-008, *Butyrivibrio*, and *Roseburia* (which produce SCFAs) at the genera level in patients with primary choledocholithiasis. Lyu *et al*[12] found that specific bacteria-producing SCFAs might participate in generating common bile duct stones.

In recent years, several groups interrogated the human biliary microbiome in recurrent bile duct stones patients, albeit in different disease states. The biliary microbiome in the recurrence of choledocholithiasis showed a significantly low diversity[17]. The composition of biliary bacteria differed significantly between primary bile duct stone patients and those with secondary bile duct stones. By contrast, several families, such as *Propionibacteriaceae*, *Sphingomonadaceae*, and *Lactobacillaceae*, were enriched in the recurrent cholelithiasis group[18]. Chen *et al*[19] analyzed the biliary microbiota of 16 patients with recurrent choledocholithiasis and 44 patients with primary choledocholithiasis. The 16S rRNA sequencing revealed a prevalence of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (all of which are intestinal bacteria) in the primary choledocholithiasis group. These data suggest that the biliary microbiota might originate from gut microbiota and access the biliary tract through the duodenal papilla. Furthermore, there was substantial enrichment of the genera *Prevotella*, *Alloprevotella*, *Nesterenkonia*, and *Pyramidobacter* (without significant alterations in microbial diversity)

compared with recurrent choledocholithiasis patients[19]. One study described the biliary microbiome in recurrent cholelithiasis[20]. Investigators compared the biliary microbiomes of patients with primary choledocholithiasis with those with recurrent choledocholithiasis. Consistently, the phyla *Proteobacteria* and *Firmicutes* predominated in both groups. Patients with recurrent choledocholithiasis showed less microbial biliary diversity (with reduced *Bacteroidetes* and *Actinobacteria* and enrichment of *Synergistetes* at the phylum level) than controls. A study considered 11 adults with recurrent choledocholithiasis and nine post-endoscopic removal patients to assess the characteristics of the biliary microbiome. The richness and diversity decreased in the recurrence group. The recurrence group had a higher relative abundance of phylum *Actinobacteria* and *Firmicutes* and a lower relative abundance of *Bacteroidetes* than the non-recurrence group[21].

These association studies demonstrate a correlation between the biliary microbiome and cholelithiasis; however, these studies sampled the microbiome from bile but not the bile duct epithelium.

The gallstone microbiome

Awareness of the gallstone microbiome arose *via* a circuitous route. The first microbial study of gallstone formation dates back decades; the authors found a lower prevalence of gallstones in germ-free mice[22]. In the 1990s, bacterial DNA was detected using PCR in all common bile duct stones, mixed cholesterol stones, and brown pigment stones[23], demonstrating the existence of bacteria in gallstone formation. In the 21st century, researchers found that 42% of patients had bacteria gallstone samples[7], and over 80% of the stone cores contained bacteria, primarily from the intestine[24]. These results were vital for assessing the connections between the microbiota and cholelithiasis.

According to scanning electron microscopy studies, there are bacterial biofilms on gallstone surfaces [25]. Bacterial biofilms are microbial communities attached to the surface or embedded in the matrix [26]. The typical surface-related biofilm originates from single planktonic cells attached to the surface. These cells divide and produce extracellular polymeric substances. Microbial communities then form the three-dimensional structure attached to the surface. Biofilms are environmental reservoirs for pathogens that correlate with infectious kidney stones, bacterial endocarditis, and airway infections in cystic fibrosis patients[27]. Studies found that biofilm formed by *Salmonellae* aggregates on the surface of human gallstones[28]. The appearance of gallstones led to a 4.5-fold increase in hyperbiofilm isolates. *Salmonella spp.* increased in persistence in bile *via* genetic alterations[29].

The gut microbiome

The gut microbiome in human health and diseases has received considerable attention. The intestinal microbiota is dominated by *Bacteroidetes*, followed by *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*[30]. Investigations of the microbial composition of cholelithiasis patients using 16S rRNA profiling identified a predominance of *Firmicutes* and, to a lesser extent, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* at the phylum level[31-33]. These findings suggest a shift in the gut microbiome from healthy individuals to cholelithiasis patients, suggesting an association with cholelithiasis pathophysiology.

The gut microbiome of cholelithiasis patients is maladjusted. Most studies showed reduced gut microbiota diversity in cholelithiasis patients[5,32]. There are alterations of the gut microbiome in cholelithiasis patients of various statuses. Song and colleagues demonstrated that *Klebsiella*, *Roseburia*, *Collinsella*, *Dialister*, and *Enterobacerin* at the genus level were significantly decreased, whereas *Streptococcus*, *Lactobacillus*, *Dorea*, *Romboutsia*, *Fusobacterium*, and *Megamonas* were over-represented in asymptomatic gallstone patients relative to controls[33]. Wu *et al*[9] compared the gut microbiota of twenty-nine patients with gallbladder stones and thirty-eight healthy controls. They found increased abundances of *Proteobacteria* and decreased abundances of *Faecalibacterium*, *Lachnospira*, and *Roseburia*; this was the first study to characterize gut microbiota dysbiosis in gallstone patients. A genome-wide search suggested an underlying link between the biliary tract core microbiome and the formation of cholesterol gallstones.

There have been attempts to utilize dysbiosis as a predictive and diagnostic tool or biomarker. Keren *et al*[34] described a significantly increased abundance of *Oscillospira* and a reduced abundance of *Roseburia* at the genus level in cholelithiasis patients compared with healthy controls. They suggested that the genera *Oscillospira* and *Roseburia* may be used in the early prediction or diagnosis of cholelithiasis as microbial biomarkers[34]. The relative abundance of the genus *Eubacterium*, which metabolizes and removes cholesterol, was lower in cholelithiasis patients. Line discriminant analysis effect size analysis showed that *Ruminococcus gnavus* predicted cholelithiasis[32]. These findings suggest that the gut microbiome is altered during cholelithiasis and that these changes may be valuable for diagnosis.

CHARACTERISTIC MICROBIOME BASED ON GALLSTONES COMPONENTS

Cholesterol gallstones

Gallstones are organic matrixes of cholesterol crystals, calcium bilirubinate, mucin, and proteins in the gallbladder or biliary tract[35]. Based on the major constituents, gallstones are classified as cholesterol gallstones (> 90%) or pigment gallstones (< 10%)[36]. Cholesterol gallstones form mixed or pure cholesterol gallstones. More than 80% of gallbladder stones are composed of pure cholesterol. Cholecystolithiasis originates primarily from the gallbladder and is composed of cholesterol or mixed gallstones, primarily cholesterol or black pigment stones. Several groups independently analyzed the human microbiome of cholelithiasis patients with different components. Bacterial diversity and several functional bacterial species of cholesterol-rich gallstones significantly decreased compared to those of pigment gallstones. These species include *Akkermansia muciniphila*, *Prevotella spp.*, *Bifidobacterium adolescentis*, *Alistipes spp.*, *Bacteroides spp.*, *Dorea spp.*, *Methanobacteria*, *Methanobrevibacter smithii*, *Ruminococcus spp.*, and *Faecalibacterium prausnitzii*[37]. There were various compositions of the gut microbiome in the mice in which a lithogenic diet-induced cholesterol gallstones. There was reduced richness, α diversity, the proportion of *Firmicutes*, and the ratio of *Firmicutes* to *Bacteroidetes* in the lithogenic diet group[38]. These findings suggest that alterations in the gut microbiome may play an essential role in forming cholesterol gallstones.

Helicobacter spp. were reported to participate in the formation of murine cholesterol gallstones. Mice fed a lithogenic diet and infected with various enterohepatic *Helicobacter spp.* showed a significantly higher prevalence of cholesterol gallstones than uninfected controls[39]. In humans, a retrospective cohort study demonstrated that patients with gallstone disease were at increased risk of *Helicobacter pylori* (*H. pylori*) infection[40].

Pigment gallstones

Pigment gallstones include brown pigment stones or black pigment stones. Brown pigment stones are associated with biliary tract infections. By contrast, black pigment stones are common in patients with hemolytic anemia, cirrhosis, and cardiac valve replacements. These findings were supported by human and animal research, which found changes in human microbiota in cholesterol gallstones. Nevertheless, there has been little attention paid to pigment gallstones. Notably, a pilot study indicated that the gallstone microbiome might participate in developing pigment stones[41]. The genera *Klebsiella* and *Enterococcus* (involved in bacterial biofilm formation) were dominant in pigment stones. These results suggest the participation of a gallstone microbiome in cholelithiasis. Kim and colleagues compared the biliary microbiota in patients with pigment common bile duct stones to other causes of biliary obstruction; there was enrichment of the genus *Enterococcus* in patients with common pigment bile duct stones[42]. These findings suggest a possible association between *Enterococcus* and pigment stone formation.

THE POTENTIAL MICROBIOTA-RELATED TRIGGERS IN CHOLELITHIASIS

The GI microbiome induces gallstone formation by regulating bile acid (BA) metabolism

Gallstones are formed when there is an imbalance of biliary cholesterol homeostasis. The primary pathophysiological defect in gallstones is the supersaturation of cholesterol in bile. Other factors include genetic factors (particularly lithogenic gene 1 and mitochondrial DNA variant), hepatic hypersecretion, gallbladder motility function obstacle, cholesteric phase transition, excessive secretion and accumulation of mucin, and excessive cholesterol[43,44]. Neutrophil extracellular DNA traps are involved in human gallstone formation and growth[45]. This evidence suggests the involvement of the GI microbiome in cholelithiasis. The alteration of the host GI microbiome modulates gallbladder motility and inflammation (especially mucin content), inhibiting cholesterol cholelithogenesis. The prevalence of gallstones in germ-free mice was higher than in specific pathogen-free mice[46], suggesting an underlying role of the GI microbiome in cholesterol cholelithogenesis.

Like gastric secretions and hydrochloric acid, bile is a bactericidal agent in the GI system[47]. The human gut microbiome is highly capable of transforming BAs. The oxidation of 3α-, 7α-, or 12α-hydroxyl groups on the steroid core, catalyzed by hydroxysteroid dehydrogenases, were the most prevalent BAs transformations[48]. A study found that 43 isolates of 41 species can modify human unconjugated BAs *in vitro*[48]. The gut microbiome chemically modifies primary BAs (*e.g.*, dehydroxylation) to affect physiology. The modified BAs are considered "secondary" BAs. The 7α-dehydroxylation of primary BAs into secondary BAs is responsible for the GI microbiota.

The genus *Clostridium* appears to be active in 7α-dehydroxylation. A study identified other strains, including *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, and *Roseburia intestinalis* at the phylum level [48]. The abundance of 7α-dehydroxylating bacteria significantly increased[32] and was 42-fold higher in gallstone subjects than in gallstone-free contrlos[49]. Antibiotic treatment significantly reduced 7α-dehydroxylation activity and cholesterol saturation[50], suggesting a possible pathogenic role for 7α-

dehydroxylating bacteria. Hu *et al*[51] found that patients with cholesterol gallstone disease showed significant enrichment of *Desulfovibrionales* at the order level compared with gallstone-free controls. Following fecal transplantation from gallstone patients into mice, the mice showed a higher prevalence of gallstones; this study suggested *Desulfovibrionales* as a microbial trigger contributing to gallstone formation.

These studies elucidated the underlying mechanisms of the biliary microbiome on gallstone formation. *Desulfovibrionales* were enriched in cholelithiasis patients. The bacterial overgrowth shifts the biliary microbiome to a cholelithogenesis phenotype. The GI microbiome, rich in *Desulfovibrionales*, induces the formation of cholesterol gallstones by regulating hepatic BA metabolism in several ways [51]. First, the secondary BAs in the cecum increase with the number of 7α-dehydroxylating bacteria. Second, the biliary microbiome regulates the expression of hepatic farnesoid X receptor (FXR)-CYP7A, inhibiting synthesis. Third, a specific microbiome promotes intestinal cholesterol absorption and secretion of canalicular cholesterol into bile[51].

Lipopolysaccharide upregulates mucins via several pathways

Mucin is a glycoprotein with high molecular weight. It protects and lubricates the ducts and lumens *in vivo*. Mucins are classified according to their structural characteristics as secreted gel-forming mucins, soluble mucins, and trans-membrane mucins. The gel-forming mucin known as MUC5AC and the trans-membrane mucin known as the multifunctional protein MUC4 participate in cell signaling due to differential expression in normal and pathophysiological conditions. For example, the abnormal expression of MUC4 and MUC5AC was detected in biliary tract cancer, whereas it is rarely detected in healthy biliary tract[52]. Yoo *et al*[53] reported that concentrations and gene expression of MUC3 and MUC5B were significantly overexpressed in a cholesterol stone group than in normal controls. Patients with gallbladder stones were subdivided according to the density of gallstones into an isopycnic group and a calcified group. The enriched expression of MUC4 was detected, and the proportion of bacteria (especially gram-positive bacteria) was positively associated with the expression of MUC4 in the calcification group[54]. Because of the function of MUC4 in adhesion, a high concentration of MUC4 may be beneficial for bacterial growth and subsequently modulate gallstone formation and calcification.

Mucin hypersecretion should be considered a requirement in gallstone formation[43]. Studies found that MUC5AC plays an essential role in hepatolithiasis formation and recurrence; there was increased expression of MUC5AC and MUC2 in hepatolithiasis patients. In another investigation, the effect of MUC5AC on hepatolithiasis formation was elucidated. MUC5AC was upregulated through several pathways. Lipopolysaccharide (LPS), a major surface component of the gram-negative bacteria, upregulated MUC5AC expression in biliary epithelial cells. LPS significantly upregulated the expression of prostaglandin E_2 (PGE_2). The expression of MUC5AC and MUC2 mRNA was induced by exogenous PGE_2. The agonist of EP4, a G-protein coupled receptor, significantly increased MUC2 and MUC5AC expression. $P38^{MAPK}$ mediates PGE_2/EP4-induced MUC2 and MUC5AC upregulation. PGE_2 induces MUC2 and MUC5AC expression through the EP4/p38MAPK pathway[55]. LPS promoted epidermal growth factor receptor activation by increasing the secretion of transforming growth factor-α, resulting in overexpression of MUC5AC. By contrast, the LPS-induced MUC5AC overexpression was abolished by inhibiting tumor necrosis factor-α converting enzyme activity[56]. Mucin hypersecretion in bile may result from the upregulation of MUC genes and result in a higher bile viscosity, retaining cholesterol crystals in the biliary tract[53].

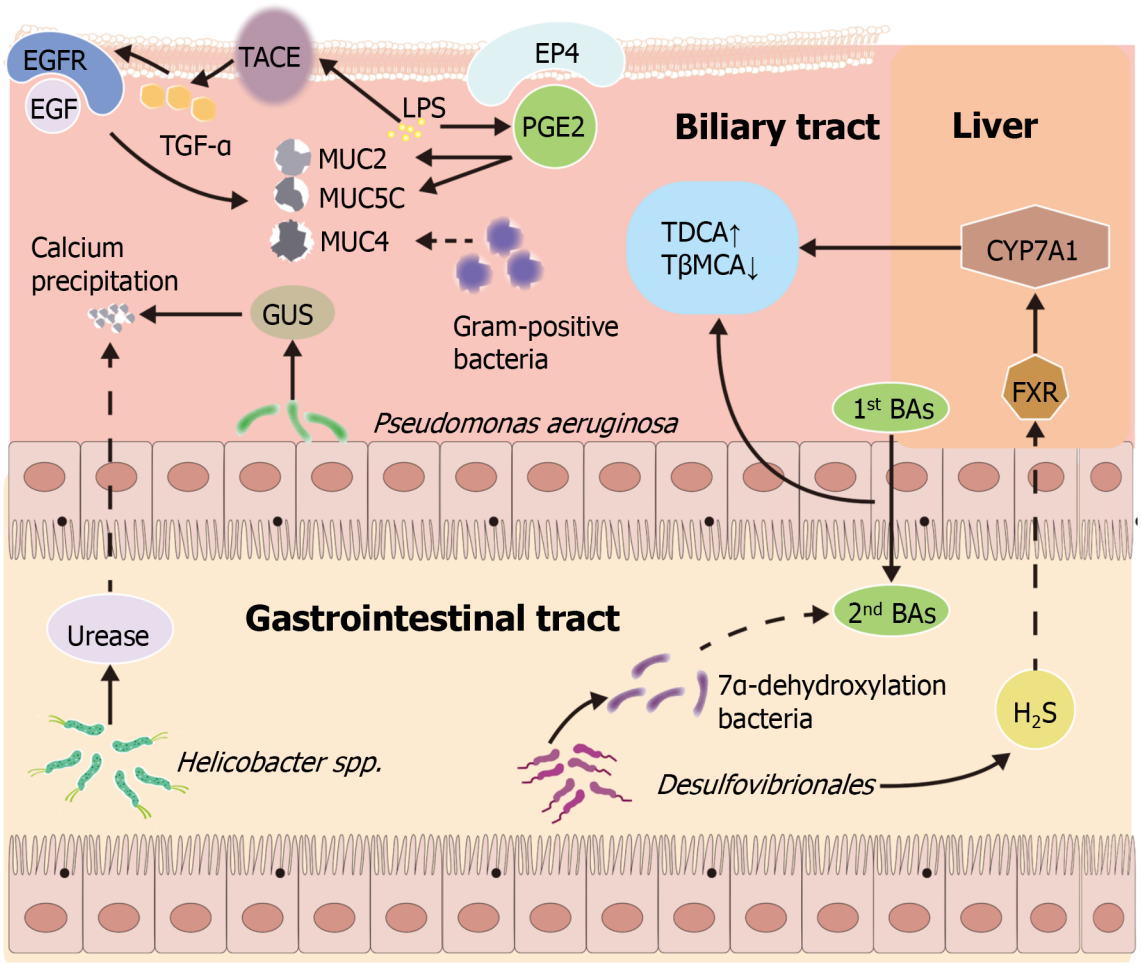
β-glucuronidase and phospholipase accelerate the precipitation of calcium bilirubinate

Bacterial enzymes, including β-glucuronidase (GUS) and phospholipase (PL), which are related to bacterial proliferation and severe infections[7], contribute to cholelithogenesis. Some bacteria produce exogenous GUS, including *Escherichia coli* and *Salmonella enterica*. GUS induces the hydrolysis of bilirubin diglucuronides to produce unconjugated bilirubin, resulting in the precipitation of calcium bilirubinate[57]. PL hydrolyzes lecithin to water-insoluble free fatty acids and lysophospholipids, enhancing the precipitation of calcium salts and mucin secretion from the biliary epithelium[58]. Nearly one-third of the cultured strains of cholesterol gallstone could secrete GUS and PLA_2[59]. GUS and PLA_2 levels in *Pseudomonas aeruginosa* (*P. aeruginosa*) strains were highest in culturable strains, suggesting that *P. aeruginosa* was involved in gallstone pathogenesis.

Helicobacter species induce gallstone formation by precipitating calcium

In addition to Desulfovibrionales, *Helicobacter spp.* are essential mediators during gallstone formation. *Helicobacter* infections (especially with *H. pylori*) positively correlate with the prevalence of chronic cholecystitis and cholelithiasis[60]. *H. pylori* is the primary pathogenic agent of chronic gastritis, gastric ulcer, and gastric cancer.

Helicobacter spp. play a unique role in the formation of murine cholesterol gallstones. Belzer *et al*[61] identified an underlying mechanism of gallstones by testing the ability of different *Helicobacter spp.* to precipitate calcium. Urease-positive *Helicobacter spp.* precipitate calcium, while urease-negative *Helicobacter* species cannot[61]. This finding suggests that gallstone formation may be induced by *Helicobacter spp.*, which precipitate calcium directly *via* urease activity. Nevertheless, there is scant evidence to



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Figure 1 The underlying microbial mechanisms of gallstone formation. The gastrointestinal microbiome may drive cholelithogenesis by: (1) The fecal microbiome enriched in *Desulfovibrionales* led to an increase of 7α-dehydroxylation bacteria, thus converting primary bile acids (Bas) to secondary BAs. Attributed to the regulation of the FXR-CYP7A1 pathway, *Desulfovibrionales* increased taurodeoxycholic acid and decreased Tauro-β-muricholic acid, which was induced by the production of H_2S; (2) Lipopolysaccharide upregulated mucins *via* TACE/TGF-α/EGFR pathway and EP4/p38MAPK pathway; (3) Gram-positive bacteria contributed to the enriched expression of mucin 4 and subsequently modulated calcification; (4) *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa* produced exogenous GUS to induce the hydrolysis of bilirubin diglucuronides, thus accelerating precipitation of calcium bilirubinate; and (5) *Helicobacter spp.* precipitated calcium *via* the urease activity. BA: Bile acids; CYP7A1: Cholesterol 7α-hydroxylase; EGFR: Epidermal growth factor receptor; EP4: E-prostanoid receptor 4; FXR: Farnesoid X receptor; GUS: β-glucuronidase; LPS: Lipopolysaccharide; MUC: Mucin; PGE2: Prostaglandin E2; PL: Phospholipase; TACE: Tumor necrosis factor-α converting enzyme; TβMCA: Tauro-β-muricholic acid; TDCA: Taurodeoxycholic acid; TGF: Transforming growth factor.

support causality in mechanisms for the GI microbiome contributing to cholelithogenesis as described above (Figure 1), and the mechanistic links between pathobionts and cholelithiasis formation require further exploration.

INFLUENCE OF HOST FACTORS ON THE MICROBIOME ASSOCIATED WITH CHOLELITHIASIS

Diet and lifestyle

The interactions and mutual influences between diet and GI microbiota are well known. Nevertheless, there is little information about the interactions between diet and biliary microbiota. The relationship between diet, biliary microbiota, and cholelithiasis is intricate. A case-control study compared the diet and biliary microbiota of patients and healthy people with cholelithiasis to identify potential associations. The authors found that the intake of dairy products was inversely associated with the relative abundance of the phylum *Bacteroidetes*, the family *Bacteroidaceae*, and the genus *Bacteroides* in bile. In contrast, seafood and meats were positively associated with the relative abundance of the family *Pasteurellaceae*[62].

Age

Epidemiological studies indicated that age, gender, pregnancy, rapid weight loss, excessive obesity, and diabetes are the primary risk factors for gallstones[63]. The prevalence of gallstone disease progressively increases with age[35]. Age impacts the composition of the human microbiome, potentially *via* the influence of health conditions, medication use, and lifestyle factors[64]. A retrospective study investigated positive bile samples from patients with biliopancreatic system diseases and found that age was positively associated with gram-negative bacterial infections and negatively related to gram-positive bacterial infections in bile[65].

History of cholecystectomy

Guidelines recommend endoscopic sphincterotomy (EST) and stone extraction for treating bile duct stones patients and cholecystectomy as the first-line treatment of symptomatic cholelithiasis[66,67]. Cholecystectomy alters the communication between the bile and intestine, altering the BA metabolism pathway and the intestinal microbiota. Several studies explored the effect of cholecystectomy on the GI microbiota. One study compared the composition of gut microbiota before and after cholecystectomy. Post-cholecystectomy patients showed significant enrichment in the phylum *Bacteroidetes*[34] and a reduction in the genus *Faecalibacterium*[68]. Other studies compared the gut microbiota of volunteers with and without cholecystectomy. Compared to controls, post-cholecystectomy patients had a lower relative abundance of the genera *Prevotella*, *Desulfovibrio*, *Barnesiella*, *Paludibacter*, and *Alistipes*[69] and a higher relative abundance of the *Blautia obeum* and *Veillonella parvula*, which are members of the phylum *Firmicutes*[70]. Reductions of *Candida albicans* and enrichments of *Candida glabrata* and *Aspergillus unassigned* were also reported[71]. These findings suggest that cholecystectomy affects the GI microbiota.

History of EST

EST is an invasive procedure recommended for treating bile duct stones. When comparing the biliary microbial composition of choledocholithiasis patients with or without a history of EST, significant differences were found between these groups; the genus *Pyramidobacter* showed positive associations with previous EST[72]. After EST, sphincter of Oddi laxity (SOL) resulted in duodenal content flow into the bile duct, altering the biliary microbiome. In two case-control studies, cholelithiasis patients with and without SOL significantly differed in the biliary microbiome[73,74]. Compared with those without SOL, cholangiolithiasis patients with SOL had increased phylotypes of family, including *Desulfovibrionaceae* and *Shewanellaceae*, and a larger abundance of *Bilophila* and *Shewanella algae*[73]. Compared with those without SOL, there was an enrichment of *Rhizobiaceae* in choledocholithiasis patients with SOL [74]. These findings suggest that SOL after EST plays a pivotal role in the bile duct microenvironment of cholelithiasis patients.

THE POTENTIAL MICROBIOME PREVENTIVE TARGETS IN CHOLELITHIASIS

Management of cholelithiasis depends on the gallstone location. Patients with gallbladder stones are usually treated with cholecystectomy and medical dissolution, whereas patients with extrahepatic bile duct stones are usually treated with EST or endoscopic papillary balloon dilation. Despite many strategies for cholelithiasis, efficacious methods of prevention are still needed. Oral administration of ursodeoxycholic acid, statins, and ezetimibe can prevent gallstones[75]. Given the cost-benefit ratios, oral administration of ursodeoxycholic acid or statins is not recommended for cholelithiasis prevention [36]. In recent years, the GI microbiome has emerged as one of the critical regulators of gallstone formation. Therefore, regulation of the host GI microbiome might be a method to prevent cholelithiasis. Some prevention strategies might include *Lactobacilli*, nanoscale iron sulfide (nFeS), and Astragalus polysaccharide.

Lactobacillus

Lactobacillus species are among the most common probiotics and are associated with health benefits, including antimicrobial activity and tumor suppression[76]. Indeed, many intervention studies using *Lactobacillus* showed promising results[77], including on cholesterol, triglyceride, and low-density lipoprotein levels[78]. Investigators demonstrated the preventive effects of *Lactobacillus* on gallstone formation in a murine model based on the probiotic's hypocholesterolemic properties. The mechanism by which *Lactobacillus* targets the GI microbiome and attenuates cholesterol gallstones was elucidated. Oh and colleagues found that *Lactobacillus acidophilus* ATCC 43121 had a hypocholesterolemic effect by decreasing the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the liver, which in turn reduces the expression of MUC5AC and MUC5B in the gallbladder[79]. Ye and colleagues found that *Lactobacillus reuteri* CGMCC 17942 and *Lactobacillus plantarum* CGMCC 14407 contribute to BA redistribution through the activation of the FXR pathway[80]. These findings suggest that supplementation with *Lactobacilli* might prevent cholesterol gallstone formation. However, further clinical

trials are needed to develop this approach as a probiotic supplement to prevent human cholelithiasis.

nFeS

Antimicrobial clinical management with bacteria or bacterial biofilms may benefit patients with gallbladder stones; nFeS is a nanomaterial with high levels of antibacterial efficacy. The evidence suggests that oral administration of nFeS supernatants significantly reduces bacterial activity and disrupts biofilm structure, inhibiting gallstone formation[81]. In a mouse model of cholelithiasis, oral administration of nFeS supernatants resulted in approximately twice the antibacterial efficacy of oral ciprofloxacin. Moreover, nFeS significantly cleared gallbladder stones compared with controls.

Astragalus polysaccharide

Astragalus polysaccharide is a natural macromolecule extracted from a standard traditional Chinese medicine known as the "Astragalus," which has immunomodulatory, anti-inflammatory, and anti-cancer effects in several diseases, including kidney stones[82], ulcerative colitis[83], constipation[84], and lung adenocarcinoma[85]. A recent study reported that Astragalus polysaccharide had beneficial effects on ameliorating the formation of cholesterol gallstones and reversing GI dysbiosis in mice[84]. Gallstone mice with Astragalus polysaccharide supplementation had a higher relative abundance of *Bacteroidota* and a lower relative abundance of *Vemucomicrobiota* at the phylum level compared to controls. These results suggest that Astragalus inhibits gallstone formation by improving intestinal microbial diversity.

CONCLUSION

These findings suggest a significant alteration of the GI microbiome in cholelithiasis patients. The GI microbiome is involved in the pathogenesis of cholelithiasis through several pathways: Biliary microbiome induces gallstone formation by regulating BA metabolism; *Helicobacter* species induce gallstone formation by precipitating calcium; LPS upregulates mucins *via* the tumor necrosis factor-α converting enzyme/transforming growth factor-α/epidermal growth factor receptor pathway and the EP4/p38MAPK pathway; GUS and PL accelerate precipitation of calcium bilirubinate. Nevertheless, the mechanisms for the GI microbiome contributing to cholelithogenesis lack evidence to support causality. The composition of the GI microbiome could be regulated in individuals with cholelithiasis by surgery, SOL, age, diet, and lifestyle. These modifiable factors for cholelithiasis may be crucial to prevent cholelithiasis. Given the regulability of the GI microbiota, studies should explore microbiome-targeting interventions for preventing cholelithiasis, including *Lactobacilli*, nFeS, and Astragalus polysaccharide.

Studies on cholelithiasis in the GI microbiome are mostly single-omics types, whereas multi-omics studies, including genome, epigenome, transcriptome, proteomics, and metabolomics, are limited. The field of the human microbiome in cholelithiasis is relatively young and limited to the bacterial microbiome (*i.e.*, there is no study of the mycobiome and virome). Existing studies are heterogeneous, possibly due to the influence of disease states, disease types, sample types and sites, gallstone components, and medical intervention. They fail accurately to characterize the structural, functional, and metabolic features of the GI microbiomes in cholelithiasis, and most lack validation cohorts. Moreover, these results do not test the specific strains, functional genes, and metabolites identified by screening validation *in vitro*, preventing in-depth studies on the pathogenesis of cholelithiasis. Consequently, longitudinal human intervention and in-depth analysis of the mechanism are needed to address the critical question of causality. If host-microbiome interactions are to be targeted as pathogenesis of cholelithiasis, well-designed mechanistic studies of the interactions between the GI microbiome and host are required. Such studies might identify causality between the GI microbiome and gallstone formation.

FOOTNOTES

Author contributions: Wang ZK established the design and conception of the paper; Dan WY and Wang ZK searched the literature and drafted the first manuscript; Wang ZK, Peng LH, and Sun G checked the manuscript and critically revised the important intellectual content in this manuscript; Yang YS contributed to manuscript revision; all authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Quantitative parameters in novel spectral computed tomography: Assessment of Ki-67 expression in patients with gastric adenocarcinoma

Li-Ting Mao, Wei-Cui Chen, Jian-Ye Lu, Han-Liang Zhang, Yong-Song Ye, Yu Zhang, Bo Liu, Wei-Wei Deng, Xian Liu

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Abstract

BACKGROUND

The level of Ki-67 expression has served as a prognostic factor in gastric cancer. The quantitative parameters based on the novel dual-layer spectral detector computed tomography (DLSDCT) in discriminating the Ki-67 expression status are unclear.

AIM

To investigate the diagnostic ability of DLSDCT-derived parameters for Ki-67 expression status in gastric carcinoma (GC).

METHODS

Dual-phase enhanced abdominal DLSDCT was performed preoperatively in 108 patients with gastric adenocarcinoma. Primary tumor monoenergetic CT attenuation value at 40-100 kilo electron volt (kev), the slope of the spectral curve (λ_{HU}), iodine concentration (IC), normalized IC (nIC), effective atomic number (Z^{eff}) and normalized Z^{eff} (nZ^{eff}) in the arterial phase (AP) and venous phase (VP) were retrospectively compared between patients with low and high Ki-67 expression in gastric adenocarcinoma. Spearman's correlation coefficient was used to analyze the association between the above parameters and Ki-67 expression status. Receiver operating characteristic (ROC) curve analysis was performed to compare

the diagnostic efficacy of the statistically significant parameters between two groups.

RESULTS

Thirty-seven and 71 patients were classified as having low and high Ki-67 expression, respectively. $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$, $CT_{100\ kev\text{-}VP}$, and Z^{eff}-related parameters were significantly higher, but IC-related parameters were lower in the group with low Ki-67 expression status than the group with high Ki-67 expression status, and other analyzed parameters showed no statistical difference between the two groups. Spearman's correlation analysis showed that $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$, $CT_{100\ kev\text{-}VP}$, Z^{eff}, and n Z^{eff} exhibited a negative correlation with Ki-67 status, whereas IC and nIC had positive correlation with Ki-67 status. The ROC analysis demonstrated that the multi-variable model of spectral parameters performed well in identifying the Ki-67 status [area under the curve (AUC) = 0.967; sensitivity 95.77%; specificity 91.89%)]. Nevertheless, the differentiating capabilities of single-variable model were moderate (AUC value 0.630 - 0.835). In addition, the nZ^{eff}_{VP} and nIC_{VP} (AUC 0.835 and 0.805) showed better performance than $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$ and $CT_{100\ kev\text{-}VP}$ (AUC 0.630, 0.631 and 0.662) in discriminating the Ki-67 status.

CONCLUSION

Quantitative spectral parameters are feasible to distinguish low and high Ki-67 expression in gastric adenocarcinoma. Z^{eff} and IC may be useful parameters for evaluating the Ki-67 expression.

Key Words: Spectral computer tomography; Quantitative parameters; Gastric carcinoma; Iodine concentration; Effective atomic number; Ki-67 expression

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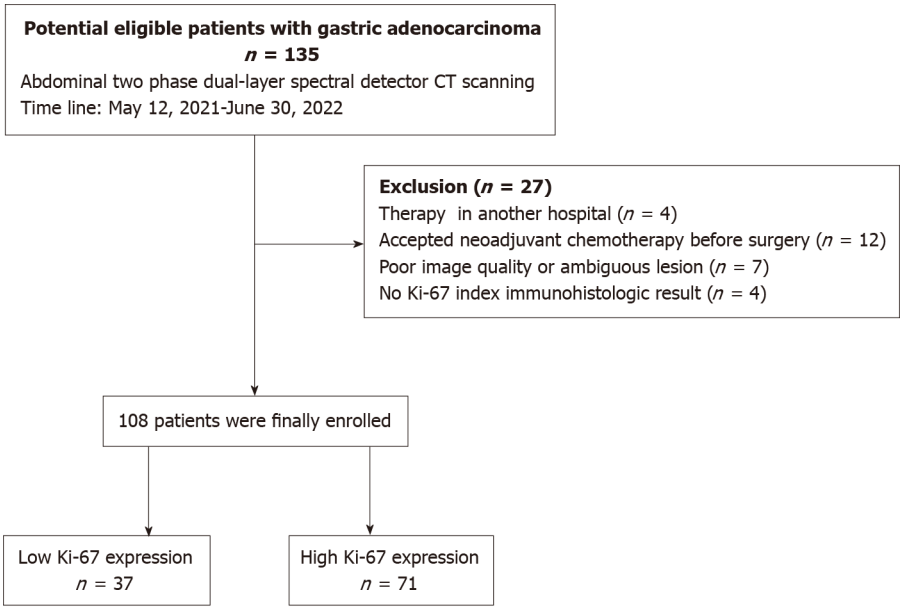
Core Tip: This is a retrospective study to preoperatively distinguish the expression of Ki-67 index based on the parameters of spectral computer tomography (CT) in patients with gastric adenocarcinoma. The CT attenuation of virtual monoenergetic images in venous phase and effective atomic number were negatively related to the expression of Ki-67, while iodine concentration exhibited positive associations with it. Multi-variable model of spectral parameters exhibited a better diagnostic efficiency than other single-variable model of spectral parameter in discriminating low and high Ki-67 expression in gastric adenocarcinoma.

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INTRODUCTION

Gastric carcinoma (GC) is the fifth commonest malignancy and the fourth most predominant cause of cancer-related mortality worldwide according to the Global Cancer Statistics 2020[1]. Although the global age-standardized rates of incidence and mortality presented a slight decrease from 1990 to 2019, China had a high incidence-mortality ratio (0.845) and five-year prevalence (27.6/100 000)[2]. A large number of GC cases are found at the advanced stage and have a relatively poor prognosis, with an overall survival rate of 25% worldwide[3]. The high proportion of tumor metastasis, intratumor heterogeneity and chemotherapeutic resistance leads to unfavorable survival outcomes in patients with GC. Conventionally, the tumor-node-metastasis (TNM) stage, histologic classification and differentiation are the major prognostic indicators for GC[4]. In addition, some oncogenic protein markers, such as antigen Ki-67, have been associated with the prognosis of GC patients[5].

Cell proliferation is a distinguishing feature of cancer. The Ki-67 protein, a nucleus-associated antigen, which is a convenient and reproducible biomarker in this process, is expressed during the cell proliferation cycle including G1, S, G2, and mitosis phases[6]. Some studies have demonstrated that Ki-67 proliferation index could be a potential indicator to predict the prognosis and identify high-risk GC cases, and was relevant to TNM stage, tumor differentiation grade, invasion depth and distant metastasis[5,7,8]. Additionally, Ki-67 index was associated with chemotherapy efficacy in the advanced GC, because cytotoxic chemotherapeutic drugs are effective against tumor cells that have entered the cell division cycle. Thus, identifying the Ki-67 status noninvasively would be beneficial for predicting



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Figure 1 The flow chart of the process of patient selection. CT: Computed tomography.

the prognosis and chemotherapeutic response in the patients with GC.

Recently, a novel dual-layer spectral detector CT (DLSDCT), which utilizes a detector-based dual-energy separation technology to acquire low- and high-energy data synchronously with two layers of detectors, makes for beam-hardening rectification, material decomposition, and image de-noising[9,10]. This system applies projection-space decomposition and generates various spectral basis images (SBIs) except for the conventional polyenergetic images, including material-specific images [iodine concentration (IC) images, virtual non-contrast images, effective atomic number (Z^{eff}) images] and energy-specific images [virtual monoenergetic images (VMIs)]. These spectral images are widely used for enhancing image contrast, improving lesion detection, characterizing materials, reducing artifact, and lowering radiation dosage[9-12]. Some studies have investigated the gastric lesions based on the IC derived from dual-energy spectral CT, such as distinguishing between malignant and benign gastric mucosal lesions[13], diagnosing GC and its histological type[14], and detecting serosal invasion of GC [15]. The latest study demonstrated that virtual monochromatic CT values were related to proliferative activity of tumor cells[16]. The Z^{eff} was correlated with Ki-67 expression in laryngeal squamous cell carcinoma[17], and could predict the vascular density of affected lesions[18]. However, only a few studies have applied the quantitative parameters [IC and normalized IC (nIC)] from spectral CT to evaluate the Ki-67 expression status in patients with gastric adenocarcinoma, but not including the CT attenuation value and Z^{eff} derived from the novel DLSDCT. Thus, the aim of this study is to explore the clinical usefulness of the quantitative parameters of novel DLSDCT in assessing the Ki-67 expression status in patients with gastric adenocarcinoma.

MATERIALS AND METHODS

Patients

This retrospective study was approved by Institutional Review Board of our hospital, with a waiver for written informed consent. From May 2021 to June 2022, 135 patients diagnosed with gastric adenocarcinoma through endoscopic biopsy underwent non-contrast and contrast-enhanced CT scans on a DLSDCT scanner (IQon Spectral CT, Philips Healthcare, Best, The Netherlands). Twenty-eight cases were excluded for the following factors: (1) Transferred to another hospital for further treatment (n = 4); (2) Accepted or required neo-adjuvant chemotherapy prior to surgery (n = 12); (3) Poor image quality or the lesion is ambiguous on the image (n = 7); or (4) Lack of the Ki-67 index immunohistologic result (n = 4). Finally, 108 patients were analyzed in this study. The detailed procedure of patient selection is shown in Figure 1.

Spectral CT imaging protocol

The interval between the DLSDCT examination and surgery was less than one week. Before CT examination, patients were required to be fast for 6-8 h and drank 800-1000 mL of water. The scan ranged from the diaphragm to the symphysis pubis in a supine position and cranio-caudal direction.

Nonionic contrast agent (Ultravist, Byer HealthCare) (370 mg/mL, 80 mL) was injected intravenously at a flow rate of 2.5 mL/s, with an automated injector (Stellant, Medrad, Byer HealthCare), following 30 mL of normal saline flushing at the same flow rate. Using bolus-tracking technique, the arterial phase (AP) scan was triggered at a threshold of 200 hounsfield unit (HU) and an additional delay of 6 s. The venous phase (VP) images were respectively collected at 35 s after injecting the contrast agent.

CT scan parameters were as follows: Tube voltage 120 kVp, automatic tube current 37-84 mAs, detector collimation 64 mm × 0.625 mm, reconstruction matrix 512 × 512. Conventional and SBI were reconstructed using the iDose 4 algorithm (Philips Healthcare). All CT images were reconstructed with a slice thickness of 1 mm and an increment of 1 mm, using a standard kernel. All image data were transferred to a workstation (IntelliSpace Portal, version 10.1, Philips Healthcare) for post-processing and analysis.

Image analysis

The image analysis was conducted by two gastrointestinal radiologists (reader 1 and reader 2, with 10 and 26 years of experience, respectively), who were blind to pathological results. The two-dimensional region-of-interest (ROI) was drawn on lesion manually, according to the following principles: (1) Polygon ROIs covered the enhanced areas of the lesions as much as possible; (2) Be careful to avoid the areas of necrosis, calcification, and vessels; (3) All lesions were measured on three consecutive axial layers by the same evaluator, and average values were calculated; and (4) The size, form, and position of the ROIs were maintained consistent between two phases images, by applying the copy-and-paste function of the workstation. A circular ROI was placed in the abdominal aorta parallel with lesion. The intraclass correlation coefficient (ICC) between the two radiologists was calculated. The final results of all ROIs were measured by the radiologist with 10 years' experience.

The following quantitative spectral parameters were automatically calculated using the post-processing software: The CT attenuation values of monochromatic images [40 kilo electron volt (kev), 70 kev and 100 kev], IC, and the Z^{eff}. Additionally, three related parameters were measured: (1) The nIC was computed as Nic = IC_{lesion}/IC_{aorta}, where IC_{lesion} and IC_{aorta} are the ICs of the lesions and abdominal aorta, which help to minimize individual variation; (2) The normalized Z^{eff} (nZ^{eff}) was counted similarly to nIC, $nZ^{eff} = Z^{eff}_{lesion}/Z^{eff}_{aorta}$; and (3) The slope of the spectral curve (λ_{HU}) was calculated as the CT attenuation values, $\lambda_{HU} = (CT_{40\,kev} - CT_{70\,kev})/30$, where $CT_{40\,kev}$ and $CT_{70\,kev}$ are the attenuation of the tumors at 40 kev and 70 kev monochromatic images, respectively[19]. Two phases (AP and VP) of CT attenuation value, IC, nIC, Z^{eff}, nZ^{eff}, and λ_{HU} were measured.

Histopathology evaluation

A pathologist with 22 years of experience (Yu Zhang) conducted the pathologic analysis. The TNM staging was determined on the basis of the American Joint Committee on Cancer (AJCC) 8th manual of gastric cancer[20]. The Ki-67 proliferation index was evaluated according to the normal immunohistochemistry process and evaluated by the pathologist blindly. The Ki-67 polyclonal antibody used (Roche #) was produced by Shanghai Rebiosci Biotech Co., Ltd. The specimens were analyzed in a high power field (× 400). The pathologist selected five fields of view randomly and each region was observed. Then 100 cells were selected in each field and the number and intensity of positively stained cells were counted. Finally, an average number of five fields were recorded. The Ki-67 indexes were categorized as low expression (< 50% positive cells) or high expression (≥ 50% positive cells), according to relevant reports[7,21].

Statistical analysis

Statistical analysis was performed using MedCalc Statistical Software version 19.4.1 (MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2020). Continuous variables were presented as the mean ± SD, and categorical variables as proportions. ICC analyses were performed with the data of 20 patients to evaluate the reliability of spectral parameters measurement. An ICC > 0.75 was considered good.

The Shapiro-Wilk test was used to test the normality of data distributions. The Student's *t*-test was employed to analyze the differences in clinical demographics and imaging parameters between low and high Ki-67 expressing status. Spearman's correlation coefficient was used to assess the correlation between the quantitative imaging parameters and the Ki-67 expression status.

Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic efficacy in classifying Ki-67 statuses. Area under the curve (AUC) and their 95% confidence intervals (CIs) were determined using unstratified bootstrap replicates 10 000 times. The optimal cutoff value was determined according to the Youden index. The difference between ROC curves were evaluated by pairwise comparison test. A two-sided *P* value of less than 0.05 was regarded statistically significant.

Table 1 Inter-reader agreement of spectral parameters measurement

Parameters	ICC (95%CI)	
	AP	VP
Z^{eff}_{lesion}	0.88 (0.71, 0.95)	0.86 (0.69, 0.94)
Z^{eff}_{aorta}	0.94 (0.85, 0.97)	0.95 (0.88, 0.98)
IC_{lesion} (mg/mL)	0.86 (0.56, 0.95)	0.87 (0.73, 0.95)
IC_{aorta} (mg/mL)	0.98 (0.94, 0.99)	0.95 (0.86, 0.98)
$CT_{40\ kev}$ (HU)	0.95 (0.88, 0.98)	0.96 (0.79, 0.99)
$CT_{70\ kev}$ (HU)	0.92 (0.78, 0.97)	0.91 (0.50, 0.98)
$CT_{100\ kev}$ (HU)	0.86 (0.69, 0.94)	0.89 (0.70, 0.96)

ICC: Intraclass correlation coefficient; Z^{eff}_{lesion} and Z^{eff}_{aorta}: The effective atomic number of lesion and aorta; $CT_{40\ kev}$, $CT_{70\ kev}$ and $CT_{100\ kev}$: The computed tomography attenuation value of 40 kilo electron volt (kev), 70 kev and 100 kev monochromatic images; IC_{lesion} and IC_{aorta}: The iodine concentration of lesion and aorta; HU: Hounsfield unit; AP: Arterial phase; VP: Venous phase; 95%CI: 95% confidence interval.

RESULTS

Patient demographics and histopathological findings

A total of 108 patients (mean age 61.9 years, range 34-85 years) were analyzed, consisting of 46 females and 62 males. The gastric tumors were located in the antrum in 48 cases (44.4%), in the corpus in 24 cases (22.2%), in the fundus in 11 cases (10.1%), in the antrum and corpus in 9 cases (8.3%), in the gastric angle in 9 cases (8.3%), and in the cardia in 7 case (6.4%).

Referring to the pathologic TNM staging of GC (AJCC 8th edition), 27 patients had gastric cancer of less than stage pT2, 32 patients had stage pT3 gastric cancer, and 49 patients had stage T4 gastric cancer. Twenty-six patients had no regional lymph node metastasis, whereas 24 patients had less than three lymph nodes invaded (pN1), 30 patients had 3-6 regional lymph nodes invaded (pN2), and 28 patients had seven or more regional lymph nodes invaded (pN3). The immunohistochemical staining results revealed that 37 cases were categorized as having low expression of the Ki-67, while 71 cases were categorized as having high expression. According to the World Health Organization grading criteria, 29 (26.8%) and 79 (73.1%) tumors were respectively classified as moderately and poorly differentiated adenocarcinoma.

Reliability of measurements

The ICC values for Z^{eff}_{lesion}, Z^{eff}_{aorta}, IC_{lesion}, IC_{aorta}, $CT_{40\ kev}$, $CT_{70\ kev}$, $CT_{100\ kev}$ in AP and VP were all more than 0.85, and the specific values are shown in Table 1.

Correlation between spectral parameters and Ki-67 status

Z^{eff} and nZ^{eff} in AP and VP presented a moderate negative correlation with Ki-67 status ($P < 0.001$), whereas the IC and nIC in AP and VP were moderately positive-correlated with Ki-67 status ($P < 0.001$). The $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$ and $CT_{100\ kev\text{-}VP}$ were weakly negative-correlated with Ki-67 status (all $P < 0.05$). However, the $CT_{40\ kev\text{-}AP}$, $CT_{70\ kev\text{-}AP}$, $CT_{100\ kev\text{-}AP}$ and $\lambda_{HU\text{-}AP}$ and $\lambda_{HU\text{-}VP}$ were not correlated with Ki-67 expression (all $P > 0.05$). The correlation coefficients and 95%CIs are shown in Table 2.

Comparison of spectral parameters between different Ki-67 status

Table 3 and Figure 2 show the results of quantitative analysis. Compared with high Ki-67 status, the low Ki-67 status had higher Z^{eff}_{AP}, Z^{eff}_{VP}, nZ^{eff}_{AP}, nZ^{eff}_{VP}, $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$ and $CT_{100\ kev\text{-}VP}$, and had lower IC_{AP}, IC_{VP}, nIC_{AP} and nIC_{VP}. Although the $CT_{40\ kev\text{-}AP}$, $CT_{70\ kev\text{-}AP}$ and $CT_{100\ kev\text{-}AP}$ of low Ki-67 status were slightly higher than that of high Ki-67 status, it was not statistically significant. Similarly, no significant differences were found in the λ_{HU} of AP and VP between the two groups. Figures 3 and 4 show the spectral parameter images of two cases with high and low Ki-67 expression, respectively.

Diagnostic performance

Table 4 summarizes the results of ROC analysis for evaluating the diagnostic performance of 12 significant spectral parameters in discriminating the Ki-67 status. For diagnosing the high Ki-67 labeling index, the Z^{eff}, nZ^{eff}, IC and nIC in AP and VP performed moderate efficiency (AUC value, ranged from 0.747 to 0.835), and there were no significant differences among the AUC values of these parameters. Nevertheless, the $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$, and $CT_{100\ kev\text{-}VP}$ showed general differentiating capabilities (AUC value 0.630, 0.631, 0.662, respectively) in differentiating low from high Ki-67 expression in GC, and the

Table 2 Correlation between spectral parameters and Ki-67 status

Parameters		Ki-67 status	
		r (95%CI)	*P* value
AP	$CT_{40\ kev}$	-0.162 (-0.362, 0.043)	0.093
	$CT_{70\ kev}$	-0.097 (-0.290, 0.102)	0.316
	$CT_{100\ kev}$	-0.074 (-0.277, 0.117)	0.449
	λ_{HU}	-0.137 (-0.328, 0.056)	0.156
	Z^{eff}	-0.427 (-0.606, -0.208)	< 0.001
	nZ^{eff}	-0.487 (-0.649, -0.318)	< 0.001
	IC	0.409 (0.228, 0.567)	< 0.001
	nIC	0.449 (0.288, 0.598)	< 0.001
VP	$CT_{40\ kev}$	-0.213 (-0.397, -0.005)	0.027
	$CT_{70\ kev}$	-0.216 (-0.407, -0.004)	0.025
	$CT_{100\ kev}$	-0.266 (-0.438, -0.096)	0.005
	λ_{HU}	-0.090 (-0.278, 0.093)	0.354
	Z^{eff}	-0.455 (-0.647, -0.215)	< 0.001
	nZ^{eff}	-0.555 (-0.692, -0.403)	< 0.001
	IC	0.405 (0.223, 0.571)	< 0.001
	nIC	0.502 (0.355, 0.651)	< 0.001

Z^{eff}_{AP} and Z^{eff}_{VP}: The effective atomic number in the arterial and venous phase; nZ^{eff}_{AP} and nZ^{eff}_{VP}: The normalized effective atomic number in the arterial and venous phase; IC_{AP} and IC_{VP}: The iodine concentration in the arterial and venous phase; nIC_{AP} and nIC_{AP}: The normalized iodine concentration in the arterial and venous phase; $CT_{40\ kev}$, $CT_{70\ kev}$ and $CT_{100\ kev}$: The computed tomography attenuation value of 40 kilo electron volt (kev), 70 kev and 100 kev monochromatic images; λ_{HU}: The slope of the spectral curve; HU: Hounsfield unit; AP: Arterial phase; VP: Venous phase; 95%CI: 95% confidence interval.

AUC values of these parameters had no statistical differences. Comparing the AUC values of CT attenuation with that of the Z^{eff}- and IC-related parameters, the AUC value of nZ^{eff}_{AP} was higher than $CT_{40\ kev-VP}$ (0.796 *vs* 0.630, $P = 0.047$), and the AUC values of nZ^{eff}_{VP} and nIC_{VP} were higher than $CT_{40\ kev-VP}$, $CT_{70\ kev-VP}$ and $CT_{100\ kev-VP}$ ($P = 0.02$, 0.01, 0.009 and 0.03, 0.01, 0.02, respectively). In addition, the multivariable model ($CT_{70\ kev-VP}$, nZ^{eff}_{AP}, nZ^{eff}_{VP}, nIC_{AP}, nIC_{VP}) was selected for most powerful parameters by multivariate logistic regression, and the model demonstrated excellent efficiency (AUC = 0.967; sensitivity 95.77%; specificity 91.89%) in discriminating high expression of Ki-67 in GC.

DISCUSSION

The recently developed DLSDCT could quantitatively map the IC and Z^{eff} of the tissue in enhanced images, and offer CT attenuation values on a wide range of VMIs. In this study, we explored the association between quantitative parameters derived from DLSDCT and the Ki-67 labeling index of gastric adenocarcinoma. Our results revealed that the $CT_{40\ kev-VP}$, $CT_{70\ kev-VP}$, $CT_{100\ kev-VP}$, and Z^{eff}-related parameters were significantly higher, but IC-related parameters were lower in the group with low Ki-67 status. Additionally, the $CT_{40\ kev-VP}$, $CT_{70\ kev-VP}$, $CT_{100\ kev-VP}$, and Z^{eff}-related parameters exhibited negative correlations with Ki-67 status, whereas IC-related parameters positively correlated with it. These results were partially in agreement with previous reports that used different spectral CT systems, which demonstrated that IC and nIC were positively associated with Ki-67 status, and the values of nIC were higher in poorly differentiated gastric adenocarcinomas significantly[22,23].

We found a negative correlation between Z^{eff} and Ki-67 labeling index, which is seemingly at variance with a previous study that Z^{eff} was positively correlated with Ki-67 expression in the laryngeal squamous cell carcinoma[17] and invasive breast cancer[24]. Z^{eff} reflects the total atomic numbers of complex or mixture of materials, and has a close relationship with fundamental properties of the elements[25]. Previous research indicated that the evaluation of Z^{eff} could be able to distinguish the different tissues showing similar attenuative properties at given energy[26]. On account of the concentrations of elements (Cl, K, Ca, Ti, Mn, Fe, Co, Cu, and Zn) are lower in the stomach cancerous tissue than normal tissue[25], gastric cancer tissue exhibits a lower Z^{eff} than its healthy counterpart[27]. It is

Table 3 Comparison of dual-layer spectral detector computed tomography-derived parameters between low and high Ki-67 expression status

	Parameters	Low Ki-67 status	High Ki-67 status	*t* value	*P* value
AP	$CT_{40\ kev}$	177.3 ± 43.9	162.3 ± 37.8	1.85	0.068
	$CT_{70\ kev}$	90.9 ± 20.1	85.5 ± 16.6	1.48	0.141
	$CT_{100\ kev}$	63.8 ± 12.7	61.7 ± 12.6	0.81	0.422
	λ_{HU}	2.88 ± 1.04	2.56 ± 0.98	1.58	0.118
	Z^{eff}	8.30 ± 0.19	8.21 ± 0.10	2.87	0.006
	nZ^{eff}	0.78 ± 0.04	0.74 ± 0.03	5.33	< 0.001
	IC (mg/mL)	1.61 ± 0.15	1.73 ± 0.19	-3.53	0.001
	nIC	0.15 ± 0.02	0.19 ± 0.04	-5.88	< 0.001
VP	$CT_{40\ kev}$	265.9 ± 61.4	237.3 ± 45.8	2.50	0.016
	$CT_{70\ kev}$	126.5 ± 33.4	108.0 ± 20.9	3.07	0.003
	$CT_{100\ kev}$	84.3 ± 18.7	73.9 ± 17.2	3.71	0.005
	λ_{HU}	4.65 ± 1.43	4.31 ± 1.15	1.33	0.185
	Z^{eff}	8.75 ± 0.24	8.62 ± 0.15	3.04	0.004
	nZ^{eff}	0.87 ± 0.04	0.83 ± 0.03	6.59	< 0.001
	IC (mg/mL)	2.62 ± 0.17	2.75 ± 0.21	-3.40	0.001
	nIC	0.41 ± 0.04	0.46 ± 0.06	-5.87	< 0.001

$CT_{40\ kev}$, $CT_{70\ kev}$ and $CT_{100\ kev}$: The computed tomography attenuation values of at 40 kilo electron volt (kev), 70 kev and 100 kev respectively; λ_{HU}: The slope of the spectral curve; Z^{eff}: Effective atomic number; nZ^{eff}: The normalized effective atomic number; IC: Iodine concentration; nIC: Normalized iodine concentration; AP: Arterial phase; VP: Venous phase.

known that deficiency or excess of certain essential trace metals is relevant to carcinogenesis of the specific organs[27,28]. The abnormal levels of these elements lead to the discrepancy of toxicity and proliferation activity of cancer cells. We hypothesize that Z^{eff} difference between high and low Ki-67 expression status is more significant due to abnormal metal concentration than tumor heterogeneity and angiogenesis, especially in case of using nZ^{eff}, which eliminate individual differences in hemodynamics.

The blood vessels of tumors are supplied by tumor angiogenesis and invasion of vessels around the tumor. The degree of tumor angiogenesis is strongly linked to tumor growth, progression, and metastasis[29,30]. Wang *et al*[31] found that the degree of CT enhancement is correlated with tumor angiogenesis and the malignancy of the tumor. Compared to CT contrast enhancement, IC can quantitatively indicate the degree of tumor neovascularization and reflect the deposition of iodine in the tissue objectively[32]. In this study, we detected significantly higher IC and nIC values in the AP and VP of the high-expression Ki-67 group, indicating a richer blood supply in these tumors. Compared to IC, the normalized parameter nIC minimized hemodynamic variations between individuals, which could be more comparable among different groups. These findings are consistent with the fact that the high proliferative activity is accompanied by abundant angiogenesis.

In our study, we found no statistical difference in the CT attenuation values at 40-100 kev (at 30 kev interval) in AP between low- and high-expression Ki-67 groups. Likewise, the λ_{HU} of AP and VP between the two groups showed no significant differences, which was different from the result reported by Cheng *et al*[22], who found that the λ_{HU} values were significantly different among the low, medium and high level Ki-67 groups in both VP and delayed phase, and had positive correlation with Ki-67 level. We deemed that the discrepancy might be attributable to the grouping method of Ki-67 index and the constituent ratio of differentiation degree of the analyzed cases was distinct from our study. It is necessary to further explore the usefulness of λ_{HU} values.

When evaluating the diagnostic performance of spectral parameters in discriminating the Ki-67 status, the results from ROC analysis demonstrated that the multi-variable model of spectral parameters performed excellent capacity, with high sensitivity and specificity. In contrast, the single-variable model of Z^{eff}- and IC-related parameters demonstrated moderate efficiency, and 40-100 kev (at 30 kev interval) in VP showed general efficiency. Therefore, the parameters derived from DLSDCT were feasible for the prediction of Ki-67 expression in the gastric adenocarcinoma, which is of great significance in predicting prognosis and guiding treatment for the patients with GC.

Table 4 Receiver operator characteristic analysis of spectral parameters in differentiating Ki-67 low expression status from high expression status

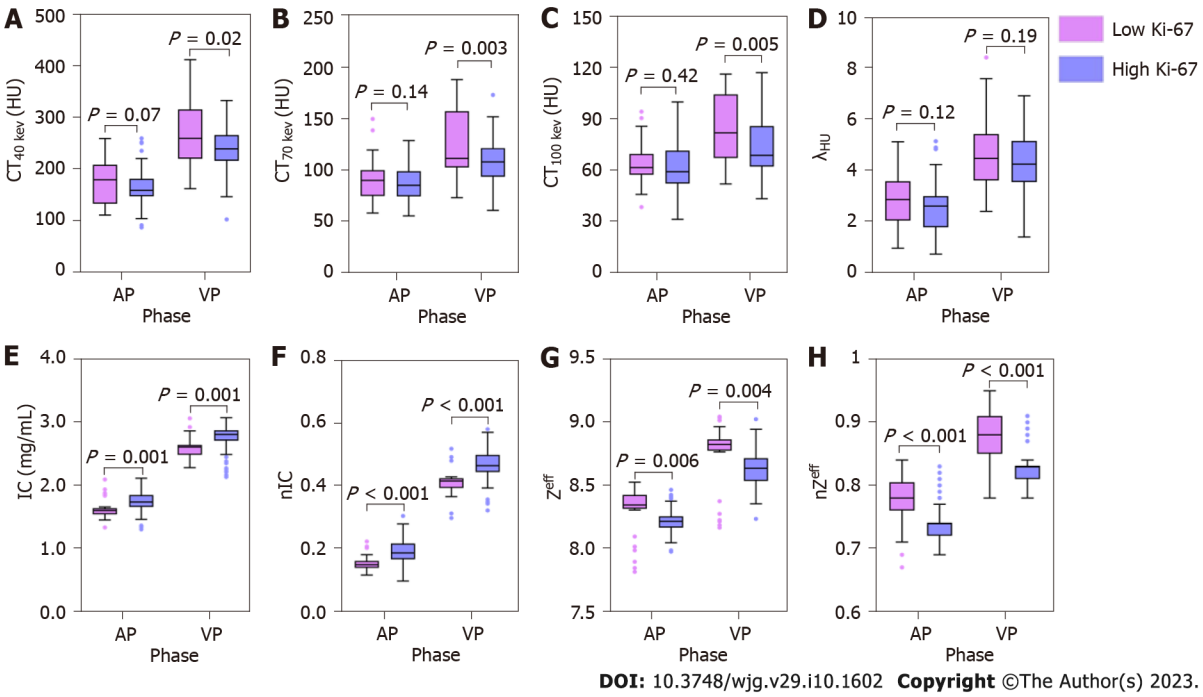
		AUC (95%CI)	TV	YI	Sen (%)	Spe (%)	PPV (%)	NPV (%)	Acc (%)
AP	Z^{eff}	0.759 (0.668, 0.836)	≤ 8.27	0.67	85.92	81.08	92.96	37.84	71.30
	nZ^{eff}	0.796 (0.707, 0.867)	≤ 0.74	0.67	80.28	86.49	85.92	54.05	75.00
	IC	0.752 (0.660, 0.830)	> 1.63	0.60	78.87	81.08	88.73	21.62	65.74
	nIC	0.773 (0.683, 0.848)	> 0.16	0.61	74.65	86.49	81.69	48.65	70.37
VP	$CT_{40\ kev}$	0.630 (0.531, 0.721)	≤ 271.2	0.26	80.28	45.95	95.77	24.32	71.30
	$CT_{70\ kev}$	0.631 (0.533, 0.722)	≤ 138.8	0.31	92.96	37.84	94.37	32.43	73.15
	$CT_{100\ kev}$	0.662 (0.565, 0.750)	≤ 89.3	0.28	81.69	45.95	87.32	32.43	68.52
	Z^{eff}	0.777 (0.687, 0.851)	≤ 8.75	0.73	88.73	83.78	92.96	29.73	74.07
	nZ^{eff}	0.835 (0.751, 0.899)	≤ 0.83	0.68	78.87	89.19	85.92	51.35	74.07
	IC	0.747 (0.654, 0.826)	> 2.65	0.63	84.51	78.38	85.92	27.03	65.74
	nIC	0.805 (0.718, 0.875)	> 0.43	0.71	78.87	91.89	85.92	37.84	69.44
Multi-parameters		0.967 (0.913, 0.992)	-	0.88	95.77	91.89	97.18	86.49	93.52

AUC: Area under the curve; TV: Threshold value; YI: Youden index; Sen: Sensitivity; Spe: Specificity; NPV: Negative predictive value; PPV: Positive predictive value; Acc: Accuracy; Z^{eff}_{AP} and Z^{eff}_{VP}: The effective atomic number in the arterial and venous phase; nZ^{eff}_{AP} and nZ^{eff}_{VP}: The normalized effective atomic number in the arterial and venus phase; IC_{AP} and IC_{VP}: The iodine concentration in the arterial and venous phase; nIC_{AP} and nIC_{AP}: The normalized iodine concentration in the arterial and venous phase; $CT_{40\ kev}$, $CT_{70\ kev}$ and $CT_{100\ kev}$: The computed tomography attenuation values of at 40 kilo electron volt (kev), 70 kev and 100 kev respectively.

There were several limitations in our study. First, this is a retrospective study in which case grouping is not random, hence, an unconscious selection bias may exist. Second, a relatively small number of patients might overstate the consequence of association. Third, the number of cases with different degrees of differentiation was disproportionate, resulting in significance hard to achieve for partially analyzed variables. Fourth, our uniform standard of enhanced scanning protocol with DLSDCT may be different from other centers, thus, the results acquired from other vendors or different scanning parameters may not be directly concluded from our results. It demands further studies to confirm and outspread our preliminary results.

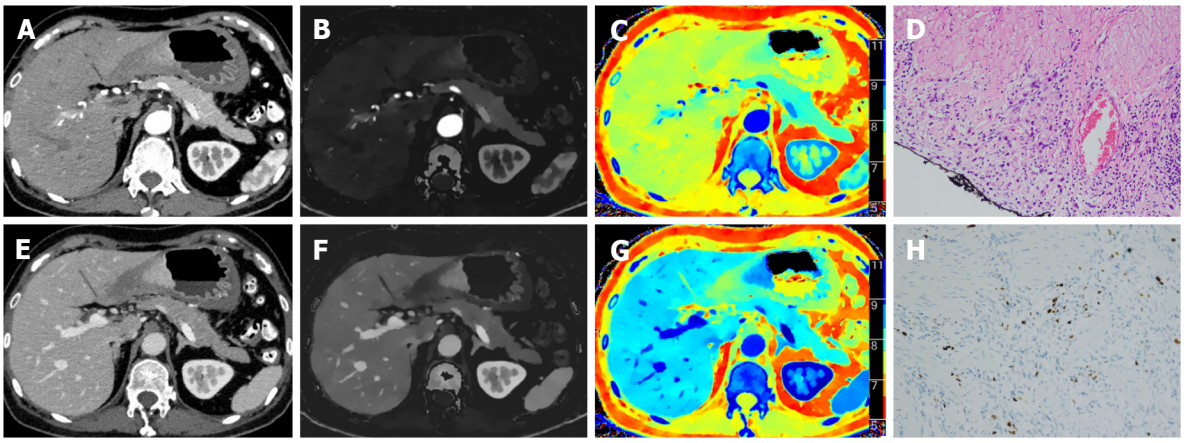
CONCLUSION

Quantitative spectral parameters are feasible to distinguish low and high Ki-67 expression in gastric adenocarcinoma. Multi-variable model of spectral parameters exhibited a better diagnostic efficiency than single-variable model of spectral parameter in discriminating low and high Ki-67 expression in gastric adenocarcinoma. Z^{eff} and IC derived from DLSDCT may be useful parameters for evaluating the Ki-67 proliferation index for gastric adenocarcinoma.



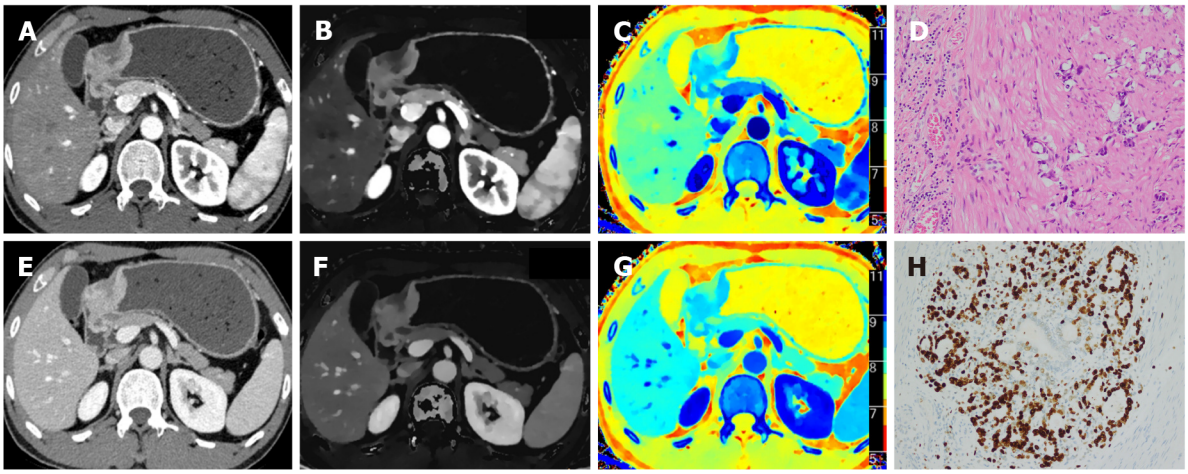
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Figure 2 Boxplots showing a comparison between low and high Ki-67 expression status demonstrated by computed tomography attenuation value of 40 kilo electron volt, 70 kilo electron volt, 100 kilo electron volt, slope of the spectral curve, iodine concentration, the normalized iodine concentration, effective atomic number, and normalized effective atomic number in both arterial phase and venous phase. A: Computed tomography attenuation value of 40 kev; B: Computed tomography attenuation value of 70; C: Computed tomography attenuation value of 100; D: The slope of the spectral curve; E: Iodine concentration; F: The normalized iodine concentration; G: Effective atomic number; H: Normalized effective atomic number. HU: Hounsfield unit; AP: Arterial phase; VP: Venous phase; IC: Iodine concentration; nIC: The normalized iodine concentration; $CT_{40\ kev}$, $CT_{70\ kev}$ and $CT_{100\ kev}$: The computed tomography attenuation value of 40 kev, 70 kev and 100 kev monochromatic images; Z^{eff}: Effective atomic number; nZ^{eff}: The normalized effective atomic number.



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Figure 3 A 57-year-old man with gastric adenocarcinoma in the antrum. A: The 70 kilo electron volt (kev) image in arterial phase (AP) show that the computed tomography attenuation value of 70 kev ($CT_{70\ kev}$) value of solid mass is 128.8 HU; B: The iodine map in AP show that the iodine concentration (IC) value of solid mass is 3.44 mg/mL; C: The effective atomic map in AP show that the effective atomic number (Z^{eff}) value of solid mass is 8.25; D: Histologic specimen shows a gastric adenocarcinoma of T4 staging in solid with HE staining (magnification, × 200); E: A 70 kev image in venous phase (VP) show that the CT_{70kev} value of solid mass is 146.9 HU; F: An iodine map in VP show that the IC value of solid mass is 3.32 mg/mL; G: An effective atomic map in VP show that the Z^{eff} value of solid mass is 8.65; H: Ki-67 immunohistochemical staining demonstrated that approximately 80% of cells were positive for nuclear staining (magnification, × 400).



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Figure 4 A 65-year-old woman with gastric adenocarcinoma in the gastric angle. A: The 70 kilo electron volt (kev) image in arterial phase (AP) show that the computed tomography attenuation value of 70 kev ($CT_{70\ kev}$) value of solid mass is 79.6 HU; B: The iodine map in AP show that the iodine concentration (IC) value of solid mass is 1.38 mg/mL; C: The effective atomic map in AP show that the effective atomic number (Z^{eff}) value of solid mass is 8.36; D: Histologic specimen shows a gastric adenocarcinoma of T4 staging in solid with HE staining (magnification, × 200); E: A 70 kev image in venous phase (VP) show that the $CT_{70\ kev}$ value of solid mass is 158.3 HU; F: An iodine map in VP show that the IC value of solid mass is 2.52 mg/mL; G: An effective atomic map in VP show that the Z^{eff} value of solid mass is 9.07; H: Ki-67 immunohistochemical staining demonstrated that approximately 40% of cells were positive for nuclear staining (magnification, × 400).

ARTICLE HIGHLIGHTS

Research background

The level of Ki-67 expression is a valuable prognostic factor in gastric cancer. However, the quantitative parameters based on the novel dual-layer spectral detector computed tomography (DLSDCT) in discriminating the Ki-67 expression status are unclear.

Research motivation

The relationship between the Ki-67 expression in gastric carcinoma (GC) and part spectral parameters (including the effective atomic number (Z^{eff}) and the monoenergetic CT attenuation) is unclear.

Research objectives

This study aimed to investigate the diagnostic ability of DLSDCT-derived parameters for Ki-67 expression status in GC.

Research methods

Dual-phase enhanced abdominal CT was performed preoperatively in 108 patients with GC. The monoenergetic CT attenuation value at 40-100 kilo electron volt (kev), the slope of the spectral curve (λ_{HU}), iodine concentration (IC), normalized IC (nIC), Z^{eff} and normalized Z^{eff} (nZ^{eff}) in the arterial phase (AP) and venous phase (VP) were retrospectively compared between the groups of low and high Ki-67 expression status. The relationship between the spectral parameters and Ki-67 expression status were analyzed, and the diagnostic efficacy of the statistically significant parameters between the two groups was compared.

Research results

The low and high Ki-67 expression groups consisted of 37 and 71 patients respectively. $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$, $CT_{100\ kev\text{-}VP}$, and Z^{eff}-related parameters were significantly higher, but IC-related parameters were lower in the low Ki-67 expression group than in the high Ki-67 expression group. $CT_{40\ kev\text{-}VP}$, $CT_{70\ kev\text{-}VP}$, $CT_{100\ kev\text{-}VP}$, Z^{eff}, and nZ^{eff} exhibited negative correlations with Ki-67 status, whereas IC and nIC positively correlated with it. The results of receiver operating characteristic analysis showed that the multi-variable model of spectral parameters performed well in identifying the Ki-67 status [area under the curve (AUC) = 0.967; sensitivity 95.77%; specificity 91.89%]. Nevertheless, the differentiating capabilities of single-variable model were moderate (AUC value from 0.630 to 0.835).

Research conclusions

Z^{eff} and IC may be useful parameters for evaluating the Ki-67 expression in GC.

Research perspectives

The spectral CT images are prospective to provide the pathological information of Ki-67 expression of GC in the future.

FOOTNOTES

Author contributions: Mao LT, Chen WC and Liu B designed the research; Lu JY, Zhang HL, Ye YS, and Deng WW collected the data; Mao LT and Liu X analyzed the data and wrote the manuscript; and all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Clinical outcomes of lenvatinib plus transarterial chemoembolization with or without programmed death receptor-1 inhibitors in unresectable hepatocellular carcinoma

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Abstract

BACKGROUND

Programmed death receptor-1 (PD-1) inhibitors have been approved as second-line treatment regimen in hepatocellular carcinoma (HCC), but it is still worth studying whether patients can benefit from PD-1 inhibitors as first-line drugs combined with targeted drugs and locoregional therapy.

AIM

To estimate the clinical outcome of transarterial chemoembolization (TACE) and lenvatinib plus PD-1 inhibitors for patients with unresectable HCC (uHCC).

METHODS

We carried out retrospective research of 65 patients with uHCC who were treated at Peking Union Medical College Hospital from September 2017 to February 2022. 45 patients received the PD-1 inhibitors, lenvatinib, TACE (PD-1-Lenv-T) therapy, and 20 received the lenvatinib, TACE (Lenv-T) therapy. In terms of the dose of lenvatinib, 8 mg was given orally for patients weighing less than 60 kg and 12 mg for those weighing more than 60 kg. Of the patients in the PD-1 inhibitor combination group, 15 received Toripalimab, 14 received Toripalimab, 14 received Camrelizumab, 4 received Pembrolizumab, 9 received Sintilimab, and 2 received Nivolumab, 1 with Tislelizumab. According to the investigators' assessment,

TACE was performed every 4-6 wk when the patient had good hepatic function (Child-Pugh class A or B) until disease progression occurred. We evaluated the efficacy by the modified Response Evaluation Criteria in Solid Tumors (mRECIST criteria). We accessd the safety by the National Cancer Institute Common Terminology Criteria for Adverse Events, v 5.0. The key adverse events (AEs) after the initiation of combination therapy were observed.

RESULTS

Patients with uHCC who received PD-1-Lenv-T therapy (n = 45) had a clearly longer overall survival than those who underwent Lenv-T therapy (n = 20, 26.8 *vs* 14.0 mo; P = 0.027). The median progression-free survival time between the two treatment regimens was also measured {11.7 mo [95% confidence interval (CI): 7.7-15.7] in the PD-1-Lenv-T group *vs* 8.5 mo (95%CI: 3.0-13.9) in the Lenv-T group (P = 0.028)}. The objective response rates of the PD-1-Lenv-T group and Lenv-T group were 44.4% and 20% (P = 0.059) according to the mRECIST criteria, meanwhile the disease control rates were 93.3% and 64.0% (P = 0.003), respectively. The type and frequency of AEs showed little distinction between patients received the two treatment regimens.

CONCLUSION

Our results suggest that the early combination of PD-1 inhibitors has manageable toxicity and hopeful efficacy in patients with uHCC.

Key Words: Lenvatinib; Programmed death receptor-1 inhibitor; Immunotherapy; Hepatocellular carcinoma; Transarterial chemoembolization; Combination therapy

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Core Tip: This retrospective research was designed to evaluate the treatment outcome and safety of transarterial chemoembolization (TACE) and lenvatinib plus programmed death receptor-1 (PD-1) inhibitors in the treatment of patients with unresectable hepatocellular carcinoma (uHCC). Patients with uHCC who underwent PD-1 inhibitors, lenvatinib, TACE therapy (n = 45) had a evidently longer overall survival than those who underrwent lenvatinib, TACE therapy (n = 20, 26.8 *vs* 14.0 mo; P = 0.027). Early combination of PD-1 inhibitors has manageable toxicity and hopeful efficacy in patients with uHCC.

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INTRODUCTION

Primary liver carcinoma is the sixth most common cancer type worldwide and leads to the third most cancer-related deaths[1]. The proportion of hepatocellular carcinoma (HCC) is approximately 80% in primary liver cancers[2]. Due to the powerful compensatory power of the liver, most patients with liver carcinoma are already in the advanced stage when they develop symptoms. Therefore, the systemic therapies of advanced HCC have attracted much attention.

Lenvatinib is an oral small molecule inhibitor of receptor tyrosine kinases [platelet-derived growth factor receptor (PDGFR), KIT, rearranged in transfection, fibroblast growth factor receptor (FGFR) 1-4 and vascular endothelial growth factor receptor (VEGFR) 1-3] that was applied for the first-line therapy of patients with unresectable HCC on the basis of the clinical outcomes from a randomized, multinational, open-label, noninferiority phase III trial[3]. In this clinical trial, lenvatinib demonstrated a better treatment effect than sorafenib. The overall survival (OS) with lenvatinib was 5 mo longer than that with sorafenib and the objective response rate (ORR) was approximately 2 times higher than that of sorafenib in hepatitis B virus (HBV) background subgroup analysis[3].

The main nutrients for liver cancer growth are supplied by the hepatic artery, which can lead to tumor ischemic necrosis by embolization[4]. Therefore, transarterial chemoembolization (TACE) is proposed as the standard therapeutic regimen for stage B HCC as classified by the Barcelona Clinic Liver Cancer (BCLC) staging system[5,6]. However, TACE also aggravates tumor hypoxia, resulting in the accumulation of hypoxia-inducible factor 1α, upregulating the expression of PDGF and VEGF,

which facilitates angiogenesis-induced collateral vessel formation, and contributes to tumor revascularization and locoregional recurrence[7-9]. Therefore, several clinical trials have made an attempt to associate TACE with systemic antiangiogenic treatment[10-12]. A randomized, multicenter prospective trial comparing TACE plus sorafenib and TACE alone in patients with liver cancer confirmed that in patients with unresectable HCC (uHCC), TACE plus sorafenib clearly improved progression-free survival (PFS) compared with TACE alone[12]. As another antiangiogenic drug, lenvatinib has significantly improved clinical efficacy in the remedy of uHCC with TACE plus lenvatinib compared with TACE monotherapy in previous studies[13,14].

Immunotherapy, as a systemic therapy that has attracted much attention in recent years, has also made significant progress in liver cancer[15]. Programmed death receptor-1 (PD-1) expresses on the surface of T cells, and is a key immunosuppressive transmembrane protein[16]. In the tumor microenvironment, cancer cells can express PD ligand 1 (PD-L1), which can bond to PD-1 to inhibit the function of T cells and reduce their killing effect on tumor cells[17]. Studies have shown that lenvatinib has a synergistic effect with immune checkpoint inhibitors (ICIs)[18]. VEGF is a highly expressed angiogenic factor in the tumor microenvironment of HCC that supports tumor growth and promotes immune rejection and is a key mediator in the immunosuppressive microenvironment[19]. Previous researches have suggested that lenvatinib can alleviate immunosuppression in the tumor microenvironment by inhibiting VEGF and can also increase T lymphocyte infiltration in the immunosuppressive microenvironment, providing an effective immunotherapeutic microenvironment for ICIs to function[20].

In conclusion, the combination of systemic and local treatment for liver cancer can synergistically kill tumors and prolong the survival of patients in various ways. Currently, PD-1 inhibitors have been applied as second-line remedy in HCC[21], and it is still worth studying whether patients can obtain clinical benefit from PD-1 inhibitors as first-line drugs combined with targeted drugs and local therapy. This retrospective research was designed to evaluate the clinical outcome and safety of TACE and lenvatinib plus PD-1 inhibitors in the treatment of uHCC and to determine whether the early combination of PD-1 inhibitors could benefit uHCC patients.

MATERIALS AND METHODS

Patients and study design

In this retrospective research, the clinical information and image data of HCC patients were collected from Peking Union Medical College Hospital from September 2017 to February 2022. uHCC was confirmed by more than two clinical experts according to the National Comprehensive Cancer Network guidelines. The main inclusion criteria were as the following: (1) Histologically or clinically confirmed HCC; (2) 1 or more measurable lesion by the modified Response Evaluation Criteria in Solid Tumors (mRECIST criteria); (3) BCLC stage A, B or C HCC; (4) Child-Pugh class scored as A (score 5-6) or B (score 7); (5) Eastern Cooperative Oncology Group performance status (ECOG-PS) score of 0-1; and (6) Prior resection or ablation was allowed. The exclusion criteria were as the following: (1) Secondary malignant tumor of liver; (2) Child-Pugh class C; and (3) Any contraindication to TACE, lenvatinib or PD-1 inhibitors.

Treatment

In this study, patients weighing less than 60 kg received a dose of 8 mg of lenvatinib, and those weighing more than 60 kg received a dose of 12 mg of lenvatinib orally once a day. Of the patients in the PD-1 inhibitor combination group, 15 received Toripalimab, 14 received Toripalimab, 14 received Camrelizumab, 4 received Pembrolizumab, 9 received Sintilimab, and 2 received Nivolumab, 1 with Tislelizumab. Camrelizumab, Sintilimab, Pembrolizumab and Tislelizumab were all given 200 mg every 3 wk. The dose of Toripalimab was 240 mg once every 3 wk. The dose of Nivolumab was 240 mg once every 2 wk.

TACE was performed under local anesthesia. After successful femoral artery puncture, 5-fluorouracil perfusion was performed through the celiac trunk. The tumor-supplying artery was superselected by a microcatheter, and chemoembolization was performed with a mixture of lipiodol and pirarubicin (25-40 mg/m^2). According to the investigators' assessment, TACE was performed every 4-6 wk when the patient had good hepatic function (Child-Pugh class A or B) until disease progression occurred.

According to Common Terminology Criteria for Adverse Events (CTCAE), v5.0, adverse events of patients during treatment were evaluated and graded. For grade 1 to grade 2 adverse events, there was no need to stop medication and symptomatic treatment was performed. If grade 3 or higher adverse reactions occur, medication should be suspended until the adverse reactions improve to grade 0-1 or baseline. For grade 4 or more adverse reactions, medication should be stopped and symptomatic treatment should be carried out actively.

Outcomes and assessments

The primary outcomes were OS and PFS. The definition of OS was the time from the first TACE therapy to death or the last follow-up.Tumor response was evaluated according to contrast-enhanced computed

tomography or magnetic resonance imaging. The definition of PFS was the time between the first TACE therapy and disease recurrence or the last follow-up. The secondary outcome was the frequency of main adverse events (AEs), which were evaluated by the CTCAE, v 5.0. The key AEs after the initiation of combination therapy were observed. We conducted follow-up every 3 wk to assess outcome variables until tumor progression, intolerable AEs, or death.

Statistical analysis

We used *T* tests to compare continuous data conforming to the normal distribution. χ^2 tests were performed to compare categorical variables. We used Mann-Whitney *U* tests to compare the continuous variables that did not conform to the normal distribution. Fisher's exact test was used when the sample size is less than 40 or the theoretical frequency was less than 1. The survival rates were evaluated by the Kaplan-Meier curve. We identified independent prognostic factors related to OS by univariate and multivariate analyses based on the Cox regression model. All statistical analyses were performed by SPSS, v 25.0.

RESULTS

Patient characteristics

We finally enrolled 65 patients with uHCC according to the criteria; 45 received the PD-1 inhibitors, lenvatinib, TACE therapy (PD-1-Lenv-T), and 20 received the lenvatinib, TACE (Lenv-T therapy) (Figure 1). The clinical information and data at baseline of all patients are shown in Table 1. The median age in the PD-1-Lenv-T group was 54 years old and in the Lenv-T group was 62 years old. At baseline, tumors were considered BCLC stage A in 6.67%, stage B in 17.78% and stage C in 75.56% of patients receiving PD-1-Lenv-T and stage A in 10%, stage B in 15% and stage C in 75% of patients receiving Lenv-T (P = 0.88). The ECOG-PSs were 0 in 57.78% and 1 in 42.22% of patients treated with PD-1-Lenv-T and 0 in 35.0% and 1 in 65.0% of patients treated with Lenv-T (P = 0.154). The numbers of TACE treatments were < 3 in 62.22% and ≥ 3 in 37.78% of patients received PD-1-Lenv-T and < 3 in 80.0% and ≥ 3 in 20.0% of patients received Lenv-T therapy (P = 0.26). The tumor numbers were 1 in 37.78% and more than 1 in 62.22% of patients who were treated with PD-1-Lenv-T and 1 in 20.00% and more than 1 in 80.00% of patients who were treated with Lenv-T (P = 0.26). Baseline characteristics of tumor burden score (TBS) group, extrahepatic metastasis rate and portal vein tumor thrombus rate were similar between the two groups. Median administration time of lenvatinib was 5.9 mo (range: 1.0-15.9 mo) and 7.8 mo (range: 1.1-28.2 mo) in the Lenv-T group and the PD-1-Lenv-T group (P = 0.07), respectively. The median injection timesof PD-1 inhibitors in the group that received PD-1 inhibitor therapy was 6 (range: 1-29).

Efficacy

The primary clinical outcomes of this research were OS and PFS. The median follow-up time for all enrolled patients was 25.2 mo [95% confidence interval (CI): 18.8-31.7]. The duration of OS and PFS for all included patients were 17.9 mo (95%CI: 11.8-24.1) and 11.0 mo (95%CI: 7.6-14.4), respectively (Figure 2). The median OS time in the PD-1-Lenv-T group was 26.8 mo (95%CI: 14.4-39.1), while that in the Lenv-T group was 14.0 mo [(95%CI: 10.3-17.8), P = 0.027] (Figures 2A and C). A remarkable median OS improvement of 12.8 mo was observed, suggesting that the PD-1-Lenv-T regimen may have advantage over the Lenv-T regimen. In addition, the median PFS time was different between the two treatment groups [11.7 mo (95%CI: 7.7-15.7) in the PD-1-Lenv-T group *vs* 8.5 mo (95%CI: 3.0-13.9) in the Lenv-T group (P = 0.028)] (Figures 2B and D).

The best tumor responses of all patients with uHCC are shown in Table 2. The ORR in the PD-1-Lenv-T group was 44.4%, which was obviously higher than the ORR of 20% in the Lenv-T group (P = 0.059) according to the mRECIST criteria (Table 2). The disease control rates (DCRs) were 93.3% in the PD-1-Lenv-T group and 64.0% in the Lenv-T group (P = 0.003). When stratified by BCLC stage, the DCR differed between the two groups (patients with BCLC stage A or B *vs* patients with BCLC stage C) (Table 3). A total of nine patients changed medications after disease progression, with two patients switching from lenvatinib to apatinib, two receiving donafenib, one receiving regofenib, and three receiving bevacizumab. Another patient stopped TACE and switched to HAIC (hepatic arterial infusion chemotherapy).

Prognostic factors for OS and PFS in the subgroups

We identified independent prognostic factors related to OS by univariate and multivariate analyses based on the Cox regression model (Figure 3). Basic clinical characteristics (gender, age, *etc.*), tumor characteristics (tumor size, tumor number, *etc.*) and treatment status (number of TACE treatments, *etc.*) were included in the analyses. Univariate analysis suggested that OS was related to the treatment option [P = 0.032, hazard ratio (HR) = 0.44, 95%CI: 0.21-0.93), ECOG-PS score (P = 0.003, HR = 0.34, 95%CI: 0.16-0.69), BCLC stage (P = 0.059, HR = 2.75, 95%CI: 0.96-7.86) and TBS group (P = 0.065, HR = 0.47,

Table 1 The baseline characteristics of included patients (*n*, %)

	Overall	Lenvatinib + TACE + PD-1	Lenvatinib + TACE	*P* value
Patient characteristics				
Number (*n*)	65	45	20	
Age, median (range), yr	57 (18-79)	54 (18-79)	62 (26-75)	0.066[1]
< 65	50 (76.92)	38 (84.44)	12 (60.00)	
≥ 65	15 (23.08)	7 (15.56)	8 (40.00)	
Gender				0.095[1]
Female	8 (12.31)	3 (6.67)	5 (25.00)	
Male	57 (87.69)	42 (93.33)	15 (75.00)	
ECOG-PS				0.154[1]
0	33 (50.77)	26 (57.78)	7 (35.00)	
1	32 (49.23)	19 (42.22)	13 (65.00)	
Etiology				0.002[1]
HBV	54 (83.08)	42 (93.33)	12 (60.00)	
HCV	4 (6.15)	2 (4.44)	2 (10.00)	
Non-HBV, non-HCV	7 (10.77)	1 (2.22)	6 (30.00)	
Hepatic cirrhosis				0.879[1]
Yes	43 (66.15)	29 (64.44)	14 (70.00)	
No	22 (33.85)	16 (35.56)	6 (30.00)	
AFP				0.551[1]
< 400	21 (32.31)	13 (28.89)	8 (40.00)	
≥ 400	44 (67.69)	32 (71.11)	12 (60.00)	
Child-Pugh score				0.095[1]
A5	48 (73.85)	30 (66.67)	18 (90.00)	
A6 or B7	17 (26.15)	15 (33.33)	2 (10.00)	
BCLC stage				1.000[1]
A or B	16 (24.62)	11 (24.44)	5 (25.00)	
C	49 (75.38)	34 (75.56)	15 (75.00)	
Tumor characteristics				
Tumor size				0.035[1]
< 7 cm	25 (38.46)	13 (28.89)	12 (60.00)	
≥ 7 cm	40 (61.54)	32 (71.11)	8 (40.00)	
Tumor number				0.260[1]
Single	21 (32.31)	17 (37.78)	4 (20.00)	
Multiple	44 (67.69)	28 (62.22)	16 (80.00)	
TBS group				0.173[1]
L	23 (35.38)	13 (28.89)	10 (50.00)	
H	42 (64.62)	32 (71.11)	10 (50.00)	
Portal vein tumor thrombus				0.108[1]
Presence	24 (36.92)	20 (44.44)	4 (20.00)	
Absence	41 (63.08)	25 (55.56)	16 (80.00)	
Extrahepatic metastasis				0.376[1]

Yes	23 (35.38)	18 (40.00)	5 (25.00)	
No	42 (64.62)	27 (60.00)	15 (75.00)	
TACE times				0.260[1]
< 3	44 (67.69)	28 (62.22)	16 (80.00)	
≥ 3	21 (32.31)	17 (37.78)	4 (20.00)	

[1]χ^2 test.
AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; ECOG-PS: Eastern Cooperative Oncology Group performance status; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PD-1: Programmed death receptor-1; TACE: Transarterial chemoembolization; TBS: Tumor burden score.

Table 2 Therapeutic efficacy in all patients, *n* (%)

	Lenvatinib + TACE + PD-1 (*n* = 45)	Lenvatinib + TACE (*n* = 20)	*P* value
CR	0 (0)	0 (0)	
PR	20 (44.4)	4 (20)	
SD	22 (48.9)	9 (45)	
PD	3 (6.7)	7 (35)	
ORR	20 (44.4)	4 (20)	0.059[1]
DCR	42 (93.3)	13 (64)	0.003[1]

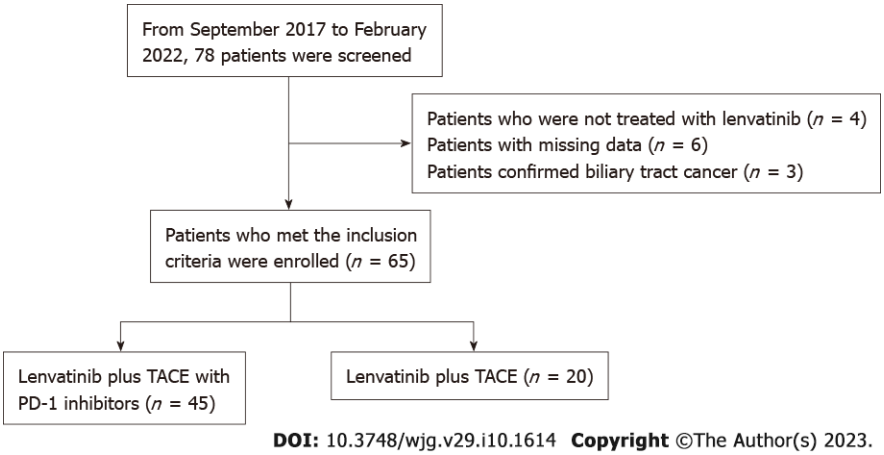
[1]χ^2 test.
[2]Fisher's exact test.
BCLC: Barcelona Clinic Liver Cancer; DCR: Disease control rate; ORR: Objective response rate; PD: Progressive disease; PR: Partial response; SD: Stable disease; PD-1: Programmed death receptor-1; TACE: Transarterial chemoembolization.

Table 3 Therapeutic efficacy in patients with stage A or B and stage C, *n* (%)

	BCLC A or B			BCLC C		
	Lenvatinib + TACE + PD-1 (*n* = 11)	Lenvatinib + TACE (*n* = 5)	*P* value	Lenvatinib + TACE + PD-1 (*n* = 34)	Lenvatinib + TACE (*n* = 15)	*P* value
CR	0 (0)	0 (0)		0 (0)	0 (0)	
PR	8 (72.7)	1 (20)		12 (35.3)	3 (20.0)	
SD	3 (27.3)	2 (40)		19 (55.9)	7 (46.7)	
PD	0 (0)	2 (40)		3 (8.8)	5 (33.3)	
ORR	8 (72.7)	1 (20)	0.077[2]	12 (35.3)	3 (20)	0.235[2]
DCR	11(100)	3 (60)	0.083[2]	31 (91.2)	10 (66.7)	0.047[2]

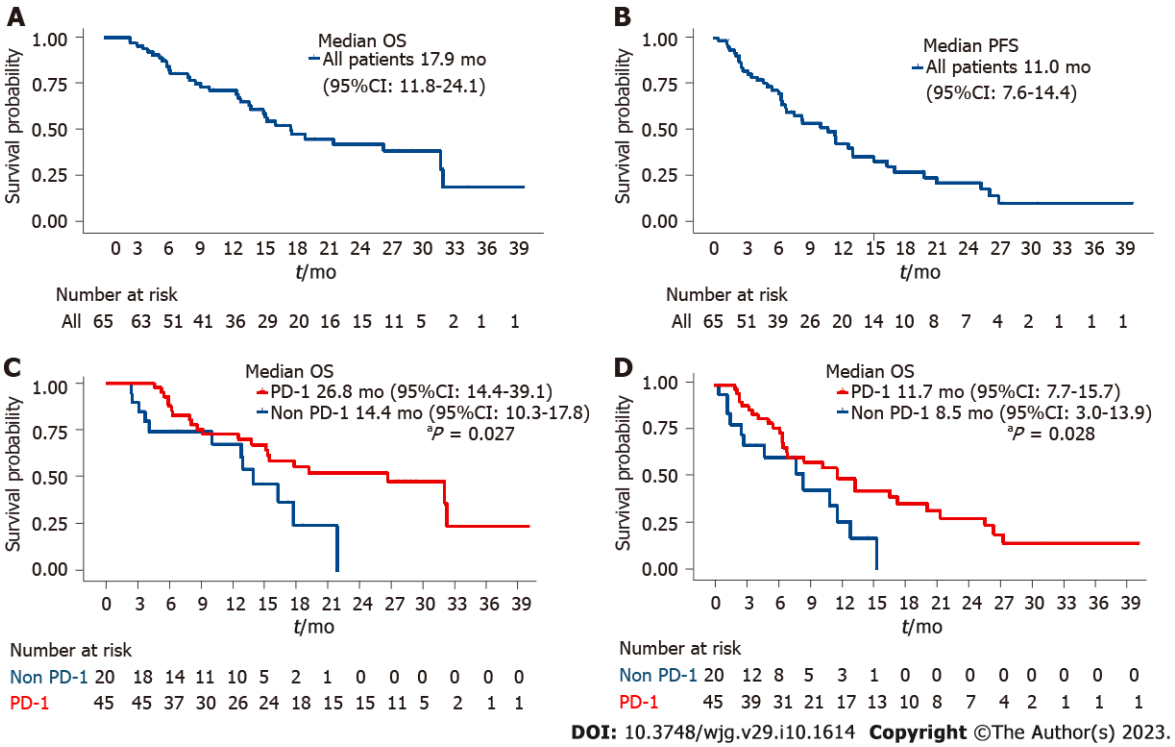
[1]χ^2 test.
[2]Fisher's exact test.
BCLC: Barcelona Clinic Liver Cancer; DCR: Disease control rate; ORR: Objective response rate; PD: Progressive disease; PR: Partial response; SD: Stable disease; PD-1: Programmed death receptor-1; TACE: Transarterial chemoembolization.

95%CI: 0.21-1.05). We subsequently included factors with a *P* value < 0.1 in the multivariate analysis and found that only the treatment option was an independent prognostic factor for OS (*P* = 0.031, HR = 0.43, 95%CI: 0.2-0.93). Similarly, univariate analysis revealed that PFS was related to the treatment option (*P* = 0.031, HR = 0.47, 95%CI: 0.23-0.93), ECOG-PS score (*P* = 0.006, HR = 2.42, 95%CI: 1.29-4.55), BCLC stage (*P* = 0.09, HR = 2.02, 95%CI: 0.9-4.56), Child-Pugh score (*P* = 0.071, HR = 1.86, 95%CI: 0.95-3.65) and number of TACE treatments (*P* = 0.017, HR = 0.44, 95%CI: 0.22-0.86). We subsequently included factors with a *P* value < 0.1 in the multivariate analysis and found that only the number of TACE treatments was an independent prognostic factor for PFS (*P* = 0.049, HR = 0.46, 95%CI: 0.21-1).



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Figure 1 Workflow of this study. TACE: Transarterial chemoembolization; PD-1: Programmed death receptor-1.



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Figure 2 The overall survival and progression-free survival times of all included patients and programmed death receptor-1-lenvatinib-transarterial chemoembolization group and lenvatinib-transarterial chemoembolization group. A: The overall survival (OS) times of all included patients; B: The progression-free survival (PFS) times of all included patients; C: The OS times of programmed death receptor-1 (PD-1)-lenvatinib-transarterial chemoembolization (TACE) group and lenvatinib-TACE group; D: The PFS times of PD-1-lenvatinib-TACE group and lenvatinib-TACE group. OS: Overall survival; PFS: Progression-free survival; CI: Confidence interval; PD-1: Programmed death receptor-1.

Safety

In total, 61 patients (93.8%) experienced AEs of any grade (Table 4). The top five most frequent treatment-related AEs in the PD-1-Lenv-T group were decreased appetite (42.2%), elevated aspartate aminotransferase (AST) (40.0%), decreased albumin (40.0%), hypertension (28.9%) and diarrhea (28.9%). In the Lenv-T group, fatigue (40.0%), decreased appetite (35.0%), and decreased albumin (30.0%) were the most frequent treatment-related AEs. Diarrhea (11.1%), decreased appetite (6.7%), elevated AST (6.7%), fatigue (6.7%), and hypertension (6.7%) were the most frequent grade 3/4 AEs in the PD-1-Lenv-T group. Decreased appetite (10.0%), decreased albumin (5.0%), diarrhea (5.0%), fatigue (5.0%), decreased platelet count (5.0%) and abdominal pain (5.0%) were the most frequent grade 3/4 AEs in the Lenv-T group. A total of three patients, two in the PD-1-Lenv-T group and one in the Lenv-T group, stopped treatment or changed treatment regimens because of intolerable AEs. Overall, the type and frequency of AEs was relatively similar between the two groups.

Table 4 Adverse events, *n* (%)

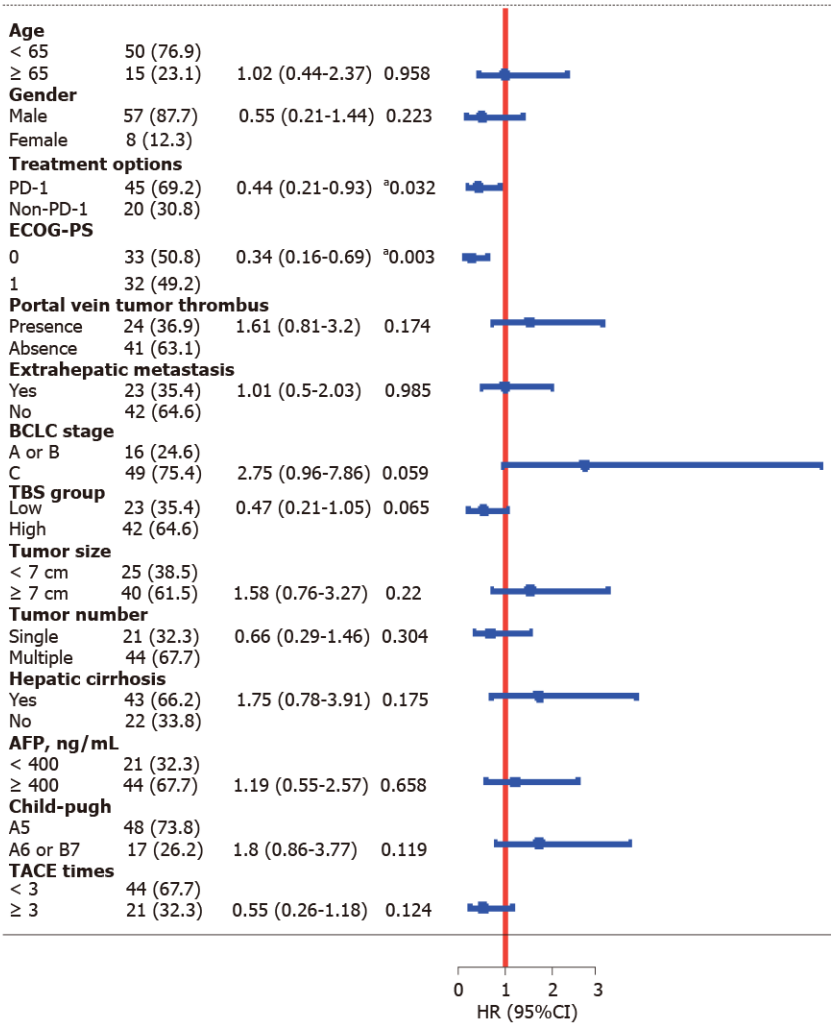
AEs	Lenvatinib + TACE + PD-1		Lenvatinib + TACE	
	All grades	Grade 3/4	All grades	Grade 3/4
Decreased appetite	19 (42.2)	3 (6.7)	7 (35.0)	2 (10.0)
Elevated AST	18 (40)	3 (6.7)	5 (25.0)	0 (0)
Decreased albumin	18 (40)	2 (4.4)	6 (30.0)	1 (5.0)
Fatigue	17 (37.8)	3 (6.7)	8 (40.0)	1 (5.0)
Diarrhoea	13 (28.9)	5 (11.1)	4 (20.0)	1 (5.0)
Hypertension	13 (28.9)	3 (6.7)	3 (15.0)	0 (0)
Elevated blood bilirubin	13 (28.9)	2 (4.4)	5 (25.0)	0 (0)
Decreased platelet count	13 (28.9)	2 (4.4)	5 (25.0)	1 (5.0)
Hypothyroidism	12 (26.7)	2 (4.4)	3 (15.0)	0 (0)
Abdominal pain	11 (24.4)	2 (4.4)	4 (20.0)	1 (5.0)
Elevated ALT	11 (24.4)	2 (4.4)	4 (20.0)	0 (0)
Rash	8 (17.8)	2 (4.4)	2 (10.0)	0 (0)
Decreased WBC	7 (15.6)	0 (0)	4 (20.0)	0 (0)
Vomiting	7 (15.6)	0 (0)	3 (15.0)	0 (0)
Hypocalcemia	6 (13.3)	0 (0)	5 (25.0)	0 (0)
Palmar-plantar erythrodysaesthesia	5 (11.1)	0 (0)	1 (5.0)	0 (0)
Nausea	4 (8.9)	0 (0)	4 (20.0)	0 (0)
Elevated WBC	4 (8.9)	0 (0)	3 (15.0)	0 (0)
Gingival bleeding	4 (8.9)	0 (0)	1 (5.0)	0 (0)
Gastrointestinal hemorrhage	4 (8.9)	1 (2.2)	0 (0)	0 (0)
Decreased weight	3 (6.7)	0 (0)	2 (10.0)	0 (0)
Dysphonia	3 (6.7)	0 (0)	2 (10.0)	0 (0)
Proteinuria	1 (2.2)	1 (2.2)	1 (5.0)	0 (0)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; AEs: Adverse events; PD-1: Programmed death receptor-1; TACE: Transarterial chemoembolization.

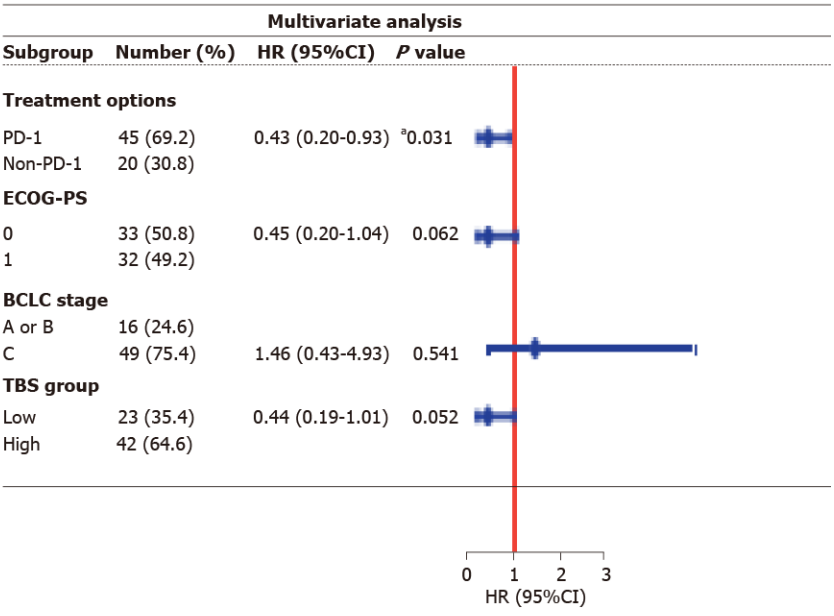
DISCUSSION

With the progress of new tyrosine kinase inhibitors and immunotherapy, individualized strategies for uHCC have improved. A recent randomized phase III trial, LAUNCH, comparing local therapy plus lenvatinib with lenvatinib monotherapy, demonstrated that TACE plus lenvatinib showed better overall survival in patients with uHCC (median OS: 17.8 *vs* 11.5 mo; HR = 0.45, $P < 0.001$)[22]. In our real-world study, patients in the TACE plus lenvatinib group had shorter OS and PFS than those in the LAUNCH trial (median OS: 17.8 *vs* 14.0 mo; median PFS: 10.6 *vs* 8.5 mo). A preclinical study showed that lenvatinib can blocke FGFR4 to reduce tumor PD-L1 expression and Treg differentiation, thus improving anti-PD-1 efficacy[18]. In addition to local therapies such as TACE, systemic therapy with PD-1 inhibitors is also being explored as a first-line therapy for uHCC. At the ESMO congress 2022, LEAP-002, a randomized phase III trial, enrolled 794 patients with advanced HCC who were not systematically treated and received lenvatinib plus pembrolizumab or lenvatinib alone in a 1:1 ratio [23]. Although the survival curve was initially higher in the Len + pembro group than in the monotherapy group approximately 15 mo after treatment, the prespecified statistical end point was not reached (median OS: 21.2 mo *vs* 19.0 mo, HR = 0.840, 95%CI: 0.708-0.997, $P = 0.0227$). However, in the subgroup analysis, HCC patients with HBV background benefited more in the Len + pembro group (HR = 0.75, 95%CI: 0.58-0.97). In our study, HBV-related HCC patients accounted for 93.33% in the PD-1-lenvatinib-TACE group, indicating that in the real world, Chinese HBV-related HCC patients are more likely to benefit from PD-1 inhibitors. This retrospective study estimated the clinical outcomes and safety of TACE in combination with lenvatinib and PD-1 inhibitors *vs* lenvatinib plus TACE in the

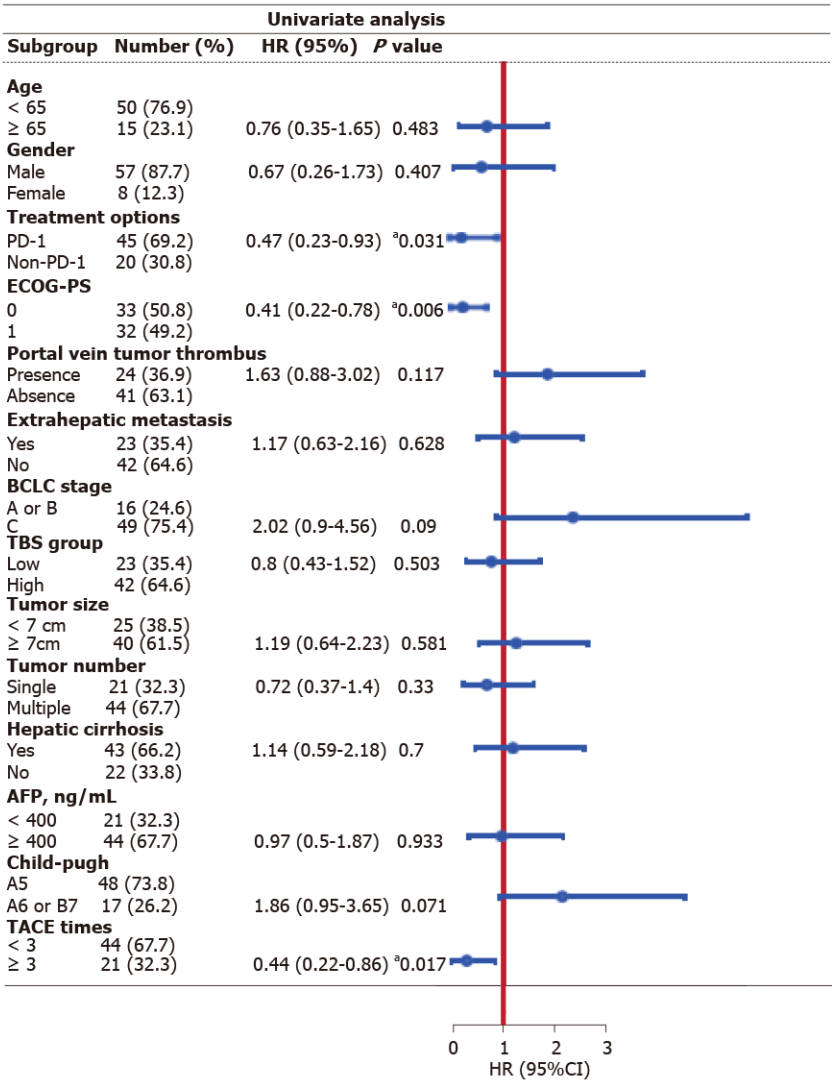
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Univariate analysis
Subgroup Number (%) HR (95%) P value
Age
< 65 50 (76.9)
≥ 65 15 (23.1) 1.02 (0.44-2.37) 0.958
Gender
Male 57 (87.7) 0.55 (0.21-1.44) 0.223
Female 8 (12.3)
Treatment options
PD-1 45 (69.2) 0.44 (0.21-0.93) a0.032
Non-PD-1 20 (30.8)
ECOG-PS
0 33 (50.8) 0.34 (0.16-0.69) a0.003
1 32 (49.2)
Portal vein tumor thrombus
Presence 24 (36.9) 1.61 (0.81-3.2) 0.174
Absence 41 (63.1)
Extrahepatic metastasis
Yes 23 (35.4) 1.01 (0.5-2.03) 0.985
No 42 (64.6)
BCLC stage
A or B 16 (24.6)
C 49 (75.4) 2.75 (0.96-7.86) 0.059
TBS group
Low 23 (35.4) 0.47 (0.21-1.05) 0.065
High 42 (64.6)
Tumor size
< 7 cm 25 (38.5)
≥ 7 cm 40 (61.5) 1.58 (0.76-3.27) 0.22
Tumor number
Single 21 (32.3) 0.66 (0.29-1.46) 0.304
Multiple 44 (67.7)
Hepatic cirrhosis
Yes 43 (66.2) 1.75 (0.78-3.91) 0.175
No 22 (33.8)
AFP, ng/mL
< 400 21 (32.3)
≥ 400 44 (67.7) 1.19 (0.55-2.57) 0.658
Child-pugh
A5 48 (73.8)
A6 or B7 17 (26.2) 1.8 (0.86-3.77) 0.119
TACE times
< 3 44 (67.7)
≥ 3 21 (32.3) 0.55 (0.26-1.18) 0.124
0 1 2 3
HR (95%CI)

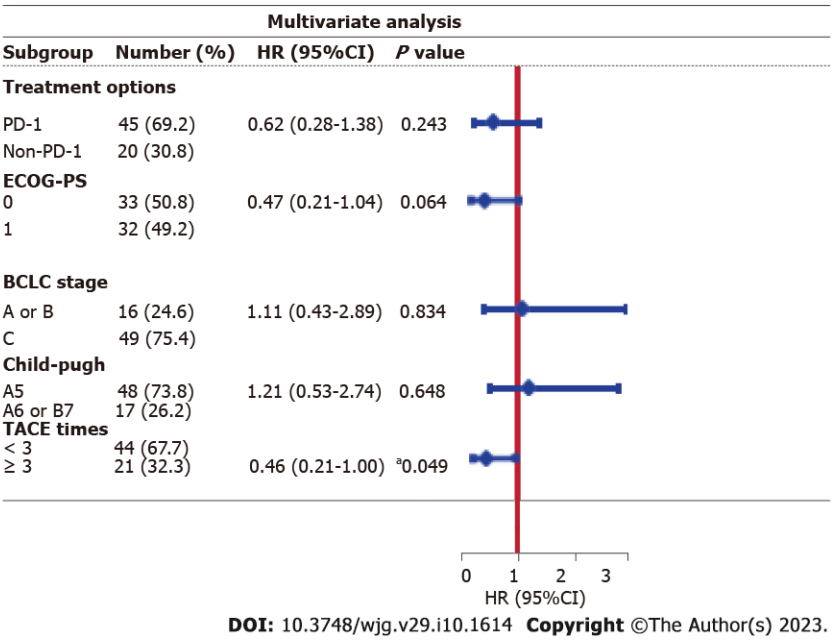
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Multivariate analysis
Subgroup Number (%) HR (95%CI) P value
Treatment options
PD-1 45 (69.2) 0.43 (0.20-0.93) a0.031
Non-PD-1 20 (30.8)
ECOG-PS
0 33 (50.8) 0.45 (0.20-1.04) 0.062
1 32 (49.2)
BCLC stage
A or B 16 (24.6)
C 49 (75.4) 1.46 (0.43-4.93) 0.541
TBS group
Low 23 (35.4) 0.44 (0.19-1.01) 0.052
High 42 (64.6)
0 1 2 3
HR (95%CI)

C



D



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Figure 3 Univariate and multivariable Cox regression analysis for overall survival and progression-free survival. A: Univariate Cox regression analysis for overall survival (OS); B: Multivariable Cox regression analysis for OS; C: Univariate Cox regression analysis for progression-free survival (PFS); D:

Multivariable Cox regression analysis for PFS. [a]*P* < 0.05. HR: Hazard ratio; CI: Confidence interval; PD-1: Programmed death receptor-1; TACE: Transarterial chemoembolization; ECOG-PS: Eastern Cooperative Oncology Group performance status; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha-fetoprotein; TBS: Tumor burden score.

remedy of uHCC. The results suggested that the PD-1-Lenv-T regimen significantly prolonged survival time in patients with uHCC, without unexpected safety-related complications.

After stratification according to BCLC stage, the ORR and DCR were 72.7% and 100%, respectively, in the PD-1-Lenv-T group of uHCC patients with BCLC stage A or B, indicating that combination early treatment with PD-1 inhibitors has a good control effect on lesions. In contrast to proportions in previous studies, 75.4% of patients in this study had BCLC stage C tumors, and 67.7% of patients had multiple tumors, which indicates greater clinical significance for the treatment of patients with advanced HCC. When identifying independent factors associated with OS, similar to previous studies, univariate log-rank analysis indicated that the treatment option (P = 0.032, HR = 0.44, 95%CI: 0.21-0.93) and ECOG-PS score (P = 0.003, HR = 0.34, 95%CI: 0.16-0.69) were associated with OS. This might be accounted for the fact that patients with a good ECOG-PS score have enough physical strength to tolerate treatment. The subsequent multivariate analysis showed that the treatment option was an independent prognostic risk factor for OS. When determining independent prognostic factors associated with PFS, the multivariate analysis suggested that only the number of TACE treatments was an independent prognostic factor for PFS (P = 0.049, 95%CI: 0.21-1); specifically, undergoing TACE ≥ 3 times prolonged the PFS of patients with uHCC. It may be that patients who respond to TACE treatment are more likely to undergo repeated TACE treatments.

According to our data, the PD-1-Lenv-T regimen performed well in terms of safety, and grade 3 or 4 AEs were rare. AEs of any grade occurred more frequently in the PD-1-Lenv-T group than in the pembrolizumab monotherapy group in the KEYNOTE-240 clinical trial, but there was no obvious difference in grade 3/4 AEs. The timely monitoring of and intervention for AEs by the treatment team also played a key role in the remedy of all patients. From the information collected, more symptomatic AEs, such as decreased appetite and fatigue, occurred with this treatment regimen. This study also exists several limitations. For example, this research was a retrospective study based on a single medical center with a limited number of patients.

CONCLUSION

In conclusion, the study results suggest that early combination treatment with PD-1 inhibitors has manageable toxicity and promising efficacy in patients with uHCC.

ARTICLE HIGHLIGHTS

Research background

Programmed death receptor-1 (PD-1) inhibitors have been approved as second-line treatment regimen in hepatocellular carcinoma (HCC), and it is still worth studying whether patients can benefit from PD-1 inhibitors as first-line drugs combined with targeted drugs and local therapy.

Research motivation

To provide more options and references for the therapy of patients with unresectable HCC (uHCC).

Research objectives

Aim to evaluate the clinical outcomes and safety of transarterial chemoembolization (TACE) and lenvatinib plus PD-1 inhibitors for patients with uHCC.

Research methods

We carried out a retrospective investigation of 65 patients with uHCC who were treated at Peking Union Medical College Hospital from September 2017 to February 2022.

Research results

Patients with uHCC who received PD-1 inhibitors, lenvatinib, TACE therapy (n = 45) had a clearly longer overall survival than those who underwent lenvatinib, TACE therapy (n = 20, 26.8 *vs* 14.0 mo; P = 0.027). The type and frequency of adverse events showed little difference between the two treatment groups.

Research conclusions

Our results suggest that the early combination of PD-1 inhibitors has manageable toxicity and promising efficacy in patients with uHCC.

Research perspectives

Patients with uHCC may obtain benefit a lot from the early combination of PD-1 inhibitors with TACE and lenvatinib.

FOOTNOTES

Author contributions: Wang YY participated in the study design and drafting of the manuscript; Yang X, Wang YC, Long JY, Sun HS, Li YR, Xun ZY, Zhang N, Xue JN and Ning C participated in data collection and statistical analysis; Zhang JW, Zhu CP, Yang XB, and Zhao HT participated in study supervision; and all authors have read and approved the final manuscript.

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Institutional review board statement: This study was approved by the Ethical Review Board at Peking Union Medical College Hospital (No.SK-2000).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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ORIGINAL ARTICLE

Retrospective Study

Clinical features, diagnosis, and treatment of Peutz-Jeghers syndrome: Experience with 566 Chinese cases

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Abstract

BACKGROUND

Peutz-Jeghers syndrome (PJS) is a clinically rare disease with pigmented spots on the lips and mucous membranes and extremities, scattered gastrointestinal polyps, and susceptibility to tumors as clinical manifestations. Effective preventive and curative methods are still lacking. Here we summarize our experience with 566 Chinese patients with PJS from a Chinese medical center with regard to the clinical features, diagnosis, and treatment.

AIM

To explore the clinical features, diagnosis, and treatment of PJS in a Chinese medical center.

METHODS

The diagnosis and treatment information of 566 cases of PJS admitted to the Air Force Medical Center from January 1994 to October 2022 was summarized. A clinical database was established covering age, gender, ethnicity, family history, age at first treatment, time and sequence of appearance of mucocutaneous pigmentation, polyp distribution, quantity, and diameter, frequency of hospitalization, fre-quency of surgical operations, *etc.* The clinical data was retrospectively analyzed using SPSS 26.0 software, with $P < 0.05$ considered statistically significant.

RESULTS

Of all the patients included, 55.3% were male and 44.7% were female. Median time to the appearance of mucocutaneous pigmentation was 2 years, and median time from the appearance of mucocutaneous pigmentation to the occurrence of abdominal symptoms was 10 years. The vast majority (92.2%) of patients underwent small bowel endoscopy and treatment, with 2.3% having serious complications. There was a statistically significant difference in the number of enteroscopies between patients with and without canceration (P = 0.004, Z = -2.882); 71.2% of patients underwent surgical operation, 75.6% of patients underwent surgical operation before the age of 35 years, and there was a statistically significant difference in the frequency of surgical operations between patients with and without cancer (P = 0.000, Z = -5.127). At 40 years of age, the cumulative risk of intussusception in PJS was approximately 72.0%, and at 50 years, the cumulative risk of intussusception in PJS was approximately 89.6%. At 50 years of age, the cumulative risk of cancer in PJS was approximately 49.3%, and at 60 years of age, the cumulative risk of cancer in PJS was approximately 71.7%.

CONCLUSION

The risk of intussusception and cancer of PJS polyps increases with age. PJS patients ≥ 10 years old should undergo annual enteroscopy. Endoscopic treatment has a good safety profile and can reduce the occurrence of polyps intussusception and cancer. Surgery should be conducted to protect the gastrointestinal system by removing polyps.

Key Words: Peutz-Jeghers syndrome; Management; Intussusception; Canceration; *STK11*

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Core Tip: Peutz-Jeghers syndrome (PJS) is a clinically rare autosomal dominant inherited disease with pigmented spots on the lips and mucous membranes and extremities, scattered gastrointestinal polyps, and susceptibility to tumors as clinical manifestations. Effective pre-ventive and curative methods are still lacking. Here we summarize our experience with 566 Chinese patients with PJS from a Chinese medical center with regard to the clinical features, diagnosis, and treatment, in order to improve the understanding of PJS in Chinese patients and improve its clinical diagnosis and treatment.

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INTRODUCTION

Peutz-Jeghers syndrome (PJS) is clinically characterized by labial mucosa and extremity terminal pigmentation and gastrointestinal multiple hamartoma polyposis[1-3]. Mucocutaneous pigmentation generally does not require specific treatment, but PJS polyposis is clinically serious. Gastrointestinal polyps cause secondary rupture, bleeding, intussusception, intestinal obstruction, abdominal pain, abdominal distension, hematochezia, and other symptoms, and their further progression causes enteric necrosis, intestinal perforation, and even cancer[4-7]. Therefore, it is essential to develop safe and effective treatments for PJS polyps. From January 1994 to October 2022, we diagnosed and treated 566 patients with PJS at the Air Force Medical Center, China, thereby accumulating some clinical experience for the management of this disease. This paper report our experience with this disease in order to explore the clinicopathological features, diagnosis, and treatment of PJS in Chinese patients.

MATERIALS AND METHODS

Materials

Patients diagnosed with PJS (ICD-9, ICD-10 disease code Q85.801) from January 1994 to October 2022 in the Air Force Medical Center's electronic medical record system were screened according to the standard reported previously[8,9]. Suspicious diagnoses and misdiagnoses were excluded, leaving a

total of 566 patients. Statistical parameters explored in this study included: (1) General information: Gender, age, ethnicity, family history, *etc.*; (2) Clinical information: Age at onset, location of mucocutaneous pigmentation, time from mucocutaneous pigmentation to the appearance of abdominal symptoms (such as abdominal pain, intestinal obstruction, and gastrointestinal bleeding), polyp distribution, polyp burden, maximum- polyp diameter, polyp pathology and canceration *etc.*; and (3) Diagnosis and treatment information: Age at initial treatment, age at follow-up, frequency of hospitalization, frequency of surgical operations, frequency of endoscopy and comorbidities, *etc.* A database of clinical parameters was created and retrospective statistical analysis was performed.

Statistical analysis

SPSS 26.0 software was used for descriptive statistical analyses. A normal testing method was applied to evaluate the quantitative data, which are expressed as the mean ± standard deviation (SD); *t*-test was used for comparisons between groups. Skewed distribution data are described as medians (interquartile ranges); the Mann-Whitney *U* test was used for comparisons between groups. Qualitative data are expressed as percentages; the comparison of proportion and correlation analysis was evaluated by the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

General data

The general information of 566 patients with PJS is shown in Table 1. As of the final follow-up age, 236 cases were married and 330 were unmarried. There were 183 cases married with children and 106 patients with healthy children after marriage; 77 children born after marriage had PJS; considering that some healthy children born to 106 patients were still too young for disease characteristics to appear, the diagnosis may not be clear, and the actual proportion of children with the disease may be higher.

Clinical data

The clinical data of 566 patients with PJS is shown in Table 2. Age distribution ranged from 0.5 to 60 years old, with a median age of 15 years old.

First treatment age: A total of 407 patients (71.9%) received initial treatment before 20 years old, and 513 (90.6%) received initial treatment before 30 years old. There was a statistically significant difference in the age of initial treatment between patients with and without a family history of PJS ($P = 0.035$, $Z = -2.114$); the age of initial treatment of patients with a family history of PJS was later than that of patients without. There was no significant difference in first treatment age according to gender ($Z = -0.105$, $P = 0.310$). There was no significant difference in first treatment age according to blood group ($H = 1.652$, $P = 0.648$). There was a significant difference in first treatment age between patients with and without malignant tumors ($P = 0.009$, $Z = -2.631$). The median age of patients with malignant tumors was significantly higher than that of patients without, which suggests that the later the age of initial treatment, the more likely the occurrence of malignancy.

Mucocutaneous pigmentation: A total of 563 cases (99.5%) had mucocutaneous pigmentation, which appeared before 7 years old in 507 cases (90.1%). As to the order of the appearance of mucocutaneous pigmentation, 402 cases (71.0%) had mucocutaneous pigmentation on both the lips and limbs, 45 (8.0%) had mucocutaneous pigmentation on the lips and then on the limbs, 4 (0.7%) had mucocutaneous pigmentation on the limbs and then on the lips, and 16 cases (2.8%) developed mucocutaneous pigmentation in an unknown order. There was a statistically significant difference in the age of the appearance of mucocutaneous pigmentation age between patients with and without a family history of PJS ($P = 0.016$, $Z = -2.415$), and the age of the appearance of mucocutaneous pigmentation in cases with a family history of PJS was significantly lower than that of patients without. There was no significant difference in the age of the appearance of mucocutaneous pigmentation and gender ($P = 0.686$, $Z = -0.404$). There was no significant difference in the age of the appearance of mucocutaneous pigmentation according to blood group ($H = 2.3$, $P = 0.512$). The age of the appearance of mucocutaneous pigmentation was positively correlated with the age of initial treatment ($r = 0.197$, $P = 0.000$). The age of initial treatment was positively correlated with small intestinal polyp burden ($r = 0.097$, $P = 0.034$) and colorectal polyp burden ($r = 0.208$, $P = 0.000$), and negatively correlated with the maximum diameter of colorectal polyps ($r = -0.120$, $P = 0.024$).

Interval time between age of mucocutaneous pigmentation and occurrence of gastrointestinal symptoms: A total of 529 cases (93.5%) had clear records; 3 cases (0.5%) had no data on gastrointestinal symptoms and 34 (6.0%) had missing data. The interval time was negatively correlated with the age of pigmentation ($r = -0.175$, $P = 0.000$). There was a statistical difference in the interval time of gastrointestinal symptoms according to the burden of colorectal polyps ($\chi^2 = 70.476$, $P = 0.015$). The greater the burden of colorectal polyps, the shorter the interval until the occurrence of gastrointestinal symptoms.

Table 1 General data of 566 patients with Peutz-Jeghers syndrome

General data	Classification	Cases	Percentage (%)
Gender	Male	313	55.3
	Female	253	44.7
Ethnic distribution	Han nationality	539	95.4
	Manchu	11	1.9
	Hui nationality	7	1.2
	Mongolian	4	0.7
	Tibetan	2	0.4
	Tujia nationality	1	0.2
	Zhuang nationality	1	0.2
	She minority	1	0.2
Family history of PJS	Yes	330	58.3
	No	236	41.7
ABO blood group	A	149	27.1
	B	175	31.8
	O	167	30.4
	AB	59	10.7
	Deletion	16	2.8
Rh blood group	Rh^+	530	97.2
	Rh^-	0	0
	Deletion	16	2.8
Marital history	Unmarried	330	58.3
	Married	236	41.7
Offspring inheritance	Yes	77	42.1
	No	106	57.9

PJS: Peutz-Jeghers syndrome.

Gastrointestinal polyps: PJS polyps were distributed in the stomach in 421 cases (74.4%), in the small intestine in 546 (96.5%), and in the colorectum in 445 (78.6%). There were 26 cases (4.6%) with gallbladder polyps, 4 (0.7%) with nasal polyps, and 2 (0.35%) with uterine polyps. Regarding pathological type, there were 433 cases of hamartoma, 75 cases of adenoma, 32 cases of canceration, and 23 cases of hyperplastic and inflammatory polyps. There was no statistically significant difference between the maximum diameter of gastric polyps and that of small intestinal polyps (χ^2 = 656.319, P = 0.991), but there was a statistically significant difference between the maximum diameter of gastric polyps and that of colorectal polyps (χ^2 = 639.396, P = 0.026). There was a statistically significant difference between the diameters of small intestinal polyps and colorectal polyps (χ^2 = 1443.082, P = 0.000). There was a statistically significant difference between the gastric polyp burden and small intestinal polyp burden (χ^2 = 1000.592, P = 0.000), between the gastric polyp burden and colorectal polyp burden (χ^2 = 468.22, P = 0.000), and between the small intestinal polyp burden and colorectal polyp burden (χ^2 = 1739.598, P = 0.000).

Endoscopic examination and treatment: Among the 566 patients in this study, 522 (92.2%) underwent enteroscopy and treatment, and a total of 1381 small intestinal enteroscopies were completed (841 transoral small intestinal enteroscopies and 533 transanal small intestinal enteroscopies). About 7236 polyps were found through endoscopy (1959 gastric polyps, 3555 small intestinal polyps and 1722 colorectal polyps). 346 polyps < 5 mm were not treated, 1489 polyps between 5-10 mm were electrocoagulated and removed by argon plasma coagulation (APC), 917 polyps between 10-20 mm were removed by endoscopic mucosal resection (EMR) and snare polypectomy (SP), and 4484 polyps > 20 mm were removed by endoscopic mucosal dissection (ESD). After endoscopic treatment, 60 cases

Table 2 Clinical data of 566 patients with Peutz-Jeghers syndrome

Clinical data	Median	(P_{25}, P_{75})	Extreme value
First treatment age	15	(9, 22)	0.5, 60
Follow-up age	26	(18, 34)	4, 65
Age of appearance of mucocutaneous pigmentation	2	(1, 4)	0, 33
Interval time between age of mucocutaneous pigmentation and occurrence of gastrointestinal symptoms	10	(5.5, 18)	0, 58
Gastric polyp burden	2	(1, 5)	1, 100
Maximum diameter of gastric polyps (mm)	8	(5, 15)	2, 80
Small intestinal polyp burden	3	(2, 8)	1, 100
Maximum diameter of small intestinal polyps (mm)	40	(25, 50)	1, 160
Colorectal polyps burden	2	(1, 5)	1, 100
Maximum diameter of colorectal polyps (mm)	30	(15, 45)	1, 120
Cumulative hospitalizations	2	(1, 3)	1, 11
Number of operations	1	(0, 2)	0, 7
Frequency of small intestinal enteroscopic examinations	2	(1, 3)	0, 12
Frequency of gastroenterographic examinations	1	(0, 2)	0, 16
Frequency of colonoscopic examinations	1	(0, 2)	0, 16

developed clinical symptoms (10 cases of abdominal discomfort, 35 cases of incomplete intestinal obstruction and 15 cases of gastrointestinal perforation and bleeding), and 47 cases (91.7%) were cured. However, 13 cases (2.3%) developed gastrointestinal perforation and bleeding, and accepted surgical operations for the perforation repair or partial resection of the small intestine. 329 patients (58.1%) underwent gastroscopy 673 times, 393 patients (69.4%) underwent colonoscopy 868 times, 38 patients (6.7%) underwent capsule endoscopy 41 times and 9 patients (1.6%) underwent gastroenterography examinations. There was a statistically significant difference in the number of small intestinal enteroscopies between patients with and without canceration (P = 0.004, Z = -2.882), which suggests that the greater number of follow-up enteroscopies, the easier the detection of polyp canceration.

Surgical treatment

Laparotomy is the most common type of surgery for the treatment of PJS, in which multiple polyps in the gastrointestinal are treated by intraoperative endoscopic resection or small incision removal, and it is necessary to removal part of the bowel in some severe cases. Due to the progress of endoscopic techniques in the treatment of PJS, we advise removing all the polyps that can be reached at the same time. Current indications for surgery include: (1) The endoscope could not reach the lesion; (2) The occurrence of acute intussusception and intestinal obstruction symptoms prevents endoscopic treatment; (3) Patients with sessile flat polyps, giant polyps, or densely distributed polyps which are difficult to remove by endoscopy; (4) Polyps pathologically confirmed to have become malignant or highly suspected of canceration under endoscopic observation; (5) The occurrence of perforation during endoscopic polyp resection or postoperative perforation which could not be treated conservatively; (6) The occurrence of major bleeding during or after endoscopic treatment which does not respond to conservative medical treatment; and (7) Patients or their family members request the disposable surgical removal of polyps. A total of 405 patients (71.6%) underwent 655 surgical procedures, 305 (75.6%) underwent abdominal surgery before the age of 35, 168 (29.7%) underwent surgical treatment twice or more, and 1 underwent surgical treatment seven times. With regard to the number of times of operation, these patients underwent nasopharyngeal polypectomy (n = 1), subtotal gastrectomy (n = 2), partial small bowel resection (n = 570), pancreaticoduodenectomy (n = 7), partialcolectomy (n = 22), left-semicolon resection (n = 2), right hemicolectomy resection (n = 4), pancolectomy (n = 11), radical resection of small bowel cancer (n = 18), radical operations for colon cancer (n = 6), radical resection of rectal carcinoma (n = 2), radical surgery for ovarian cancer (n = 3), radical hysterectomy (n = 3), radical resection of pulmonary carcinoma (n = 3), and modified radical mastectomy (n = 1). There was a statistically significant difference in the number of operations between patients with and without cancer (P = 0.000, Z = -5.127), and patients with cancer required more surgery.

Drug therapy

Of nine patients who were treated with oral celecoxib capsules 400 mg once daily after small intestinal

enteroscopies, three developed allergic reactions and three developed hematochezia, for which the treatment was ceased. Two patients completed a 6-mo course of treatment and one completed a 9-mo course of treatment. The reexamination of small intestinal enteroscopies showed that the drug therapy was effective in only one case. Traditional Chinese medicine (TCM) was used in 30 cases and was shown to inhibit the growth of some small polyps < 1 cm, but its inhibitory effects on the growth of polyps ≥ 1 cm were unsatisfactory, with an overall effective rate of only 40.0%.

Follow-up

As of the final follow-up date of this study, 361 patients were followed more than twice at our hospital, and no death occurred. A total of 338 cases (189 males and 149 females with a median age of 27.5 years) had previously or concurrently developed intussusception, intestinal obstruction, or intestinal perforation. Kaplan-Meier survival analysis showed that, at the age of 40 years, the cumulative risk of intussusception in PJS patients could reach 72.0%, and at 50 years, the cumulative risk of intussusception could reach 89.6% (Figure 1A). Forty-six patients had malignant tumors (27 males and 19 females with a median age of 36.5 years); there were 18 cases of small bowel cancer, 8 cases of colorectal cancer, 6 cases of duodenal cancer, 3 cases of ovarian cancer, 3 cases of cervical cancer, 3 cases of lung cancer, 2 cases of gastric cancer, and 1 case each of cholangiocarcinoma, breast cancer, and nasopharyngeal cancer. Kaplan-Meier survival analysis showed that, at the age of 50 years, the cumulative risk of cancer in PJS patients could reach 49.3%, and at 60 years, the cumulative risk of cancer could reach 71.7% (Figure 1B).

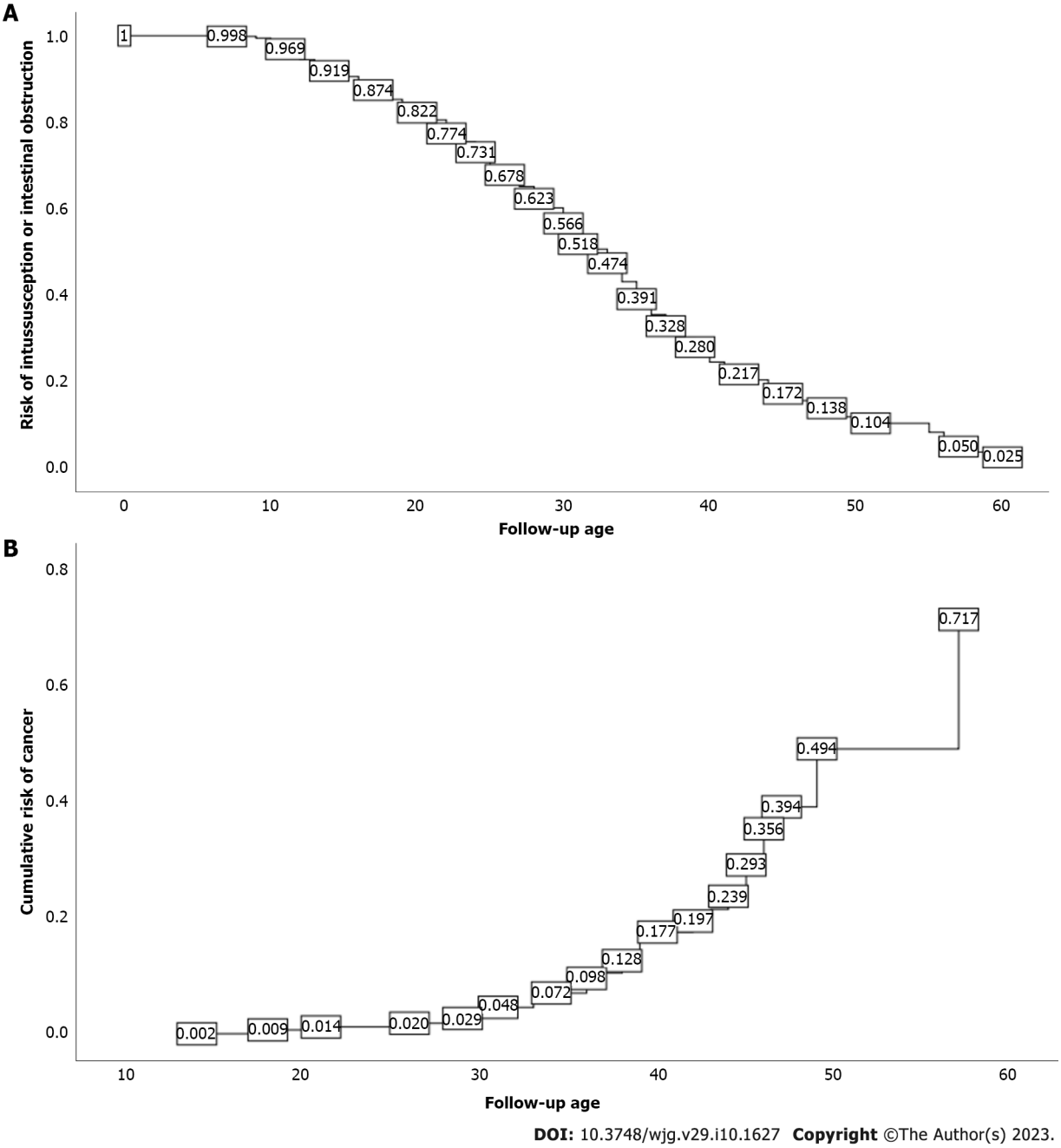
DISCUSSION

This study found that 90.1% of PJS patients developed mucocutaneous pigmentation before the age of 7 years. The median time between the appearance of mucocutaneous pigmentation and the occurrence of abdominal symptoms was 10 years. Therefore, as PJS patients are often outpatients undergoing dermatological and stomatological treatment for mucocutaneous pigmentation, which is easy for doctors unfamiliar with the disease to misdiagnose, and valuable early treatment opportunities may thus be missed, clinicians should be more vigilant. PJS that does not cause the malignant transformation of mucocutaneous pigmentation on the lips and limbs generally does not need to be treated[10]. Some scholars used a 755 nm picosecond laser to treat mucocutaneous pigmentation on the lips, which showed a reduction in pigmentation of 50%-75% after 3 mo, and good recovery after surgery[11].

At present, there is no effective treatment for PJS polyps. Some scholars used everolimus (mechanistic target of rapamycin inhibitor, a derivative of rapamycin) to treat two PJS patients, and the results showed that pancreatic cancer progressed after 2 mo in one patient, while the other refused to continue treatment due to severe toxicity, so the trial was discontinued[12]. One study[13] found that patients with PJS (2/6) responded well to celecoxib and had fewer gastric polyps, suggesting that COX-2 inhibitors may be beneficial in PJS therapy. However, in our study, celecoxib capsules were used to prevent polyps, and it was effective in only one case. This suggests that COX-2 inhibitors may not be ideal drugs for PJS therapy, as they had a high proportion of side effects (6/9) such as allergic reaction and gastrointestinal bleeding. We also treated 30 PJS patients with TCM, which had an effective rate for small polyps of only 40%, while being basically ineffective for large polyps. This study showed that PJS polyps could be distributed in the stomach (74.4%), small intestine (96.5%), and colorectum (78.6%). The burden and diameter of small intestine polyps were much higher than those of stomach polyps, which was the root cause of intussusception and intestinal obstruction. We found that some PJS patients had concurrent gallbladder polyps, nasal polyps, cervical polyps, *etc*. Whether these are the extra-gastrointestinal manifestations of PJS polyps still needs to be supported by pathological evidence. Endoscopic treatment methods for PJS polyps include EMR and ESD[14,15]. Our study showed that the incidence of gastrointestinal bleeding, perforation, and other serious complications during colonoscopy and endoscopic treatment was 2.3%. Overall, the endoscopic treatment of PJS polyps is safe and feasible.

Due to gastrointestinal polyps occurring in multiple places, patients often undergo multiple laparotomy operations, which can cause severe intraperitoneal adhesion. Laparoscopic surgery is difficult and not recommended as a routine treatment method for PJS. Laparoscopic surgery and small intestinal endoscopic surgery could be used for abdominal exploration or patients with moderate abdominal adhesions. Laparoscopic perforation repair or hemostatic suture can be used as adjuvant treatments for endoscopic complications when removing large localized polyps and dense intestinal segments of polyps with perforation or bleeding during endoscopic treatment.

Open surgery for PJS patients should be explored to determine the distribution of polyps in the whole gastrointestinal tract. Following the principle of organ protection, it is recommended to perform intestinal adhesion release and try to avoid large sections of intestinal resection causing short bowel syndrome. Multiple small incisions can be made in the intestinal wall to remove all palpable polyps, but when encountering necrotic, perforated, or dense bowel polyps, bowel segment resection is recommended. For patients diagnosed with malignant transformation, radical resection and regional lymph node dissection should be performed on cancerous intestinal segments.

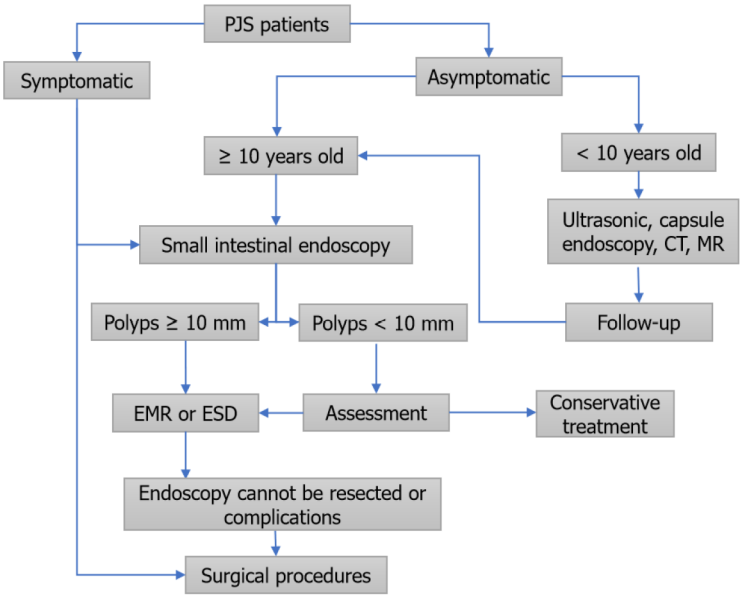


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Figure 1 Cumulative risk. A: Intussusception; B: Cancer.

PJS is a tumor-susceptible syndrome[16-18], and it has been previously reported that PJS polyps have a evolvement sequence of hamartoma-adenoma-cancer[19]. In our study, 46 cases were pathologically confirmed to have a malignant tumor, of which 35 (76.1%) were malignant transformation of PJS polyps and the rest were breast cancer, cervical cancer, lung cancer, ovarian cancer, nasopharyngeal cancer, and cholangiocarcinoma. The cumulative cancer risk of PJS patients reached 49.3% at the age of 50 years and 71.7% at the age of 60 years. Considering the short follow-up time for some cases, the actual cumulative risk of carcinogenesis may be higher. For PJS patients, we should also pay attention to their mental health, especially adolescent PJS patients. With the deepening of their understanding of PJS, *e.g.*, it is a genetic disease which requires long-term hospitalization, they may gradually develop the psychology of hating their parents and retaliating against society. We also found that PJS patients have different degrees of concern about the risk of cancer, which reminds us that we should strengthen psychological counseling and provide professional interpretation for PJS patients to reduce their anxiety and depression, and where necessary, psychologists should be involved in the treatment process of PJS patients.

Our study showed that there was a statistically significant difference in the age of the occurrence of mucocutaneous pigmentation between patients with and without a family history of PJS ($P = 0.016$, $Z = -2.415$). As such, we suggest that suspected or already confirmed PJS patients should undergo next



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Figure 2 Diagnosis and treatment process of Peutz-Jeghers syndrome patients. PJS: Peutz-Jeghers syndrome; CT: Computed tomography; MR: Magnetic resonance imaging; EMR: Endoscopic mucosal resection; ESD: Endoscopic mucosal dissection.

generation sequencing, which can reliably quantify the incidence, penetrance, mutation type, and expression of PJS. When adult patients are pregnant, they can receive preimplantation genetic testing or preimplantation genetic screening to predict whether there is a correlation with *STK11* or other gene mutation[20,21]. If screening finds that the fetus is a carrier of the *STK11* gene mutation, the parents can terminate the pregnancy; thus, this approach makes it possible to have healthy children born.

At present, there is no unified protocol for the follow-up of PJS polyps. Our study showed that the occurrence time of mucocutaneous pigmentation in most patients is earlier than the occurrence time of gastrointestinal symptoms, which is a good window period in which to intervene in the development of polyps. We suggest that PJS patients should start endoscopic examinations when they are 10 years old, and the endoscopic treatment of gastrointestinal polyps ≥ 10 mm in diameter should be performed to prevent intussusception and intestinal obstruction. The whole gastrointestinal tract should be explored by oral and transanal small intestinal enteroscopy as far as possible. Cold forceps polypectomy and cold SP or APC can be used on polyps < 10 mm which are sessile or flat; combined with high-frequency electroresection, effective eradication can be achieved without complications such as bleeding and perforation. If small intestinal enteroscopy exploration finds moderate intussusception, incomplete intestinal obstruction, and a perforation area < 1 cm, conservative treatment methods can be used, such as fasting, fluid infusion, gastrointestinal decompression, endoscopic balloon reduction, or perforation repair with titanium clips. If polyps ≥ 10 mm are detected, EMR or ESD will be required to remove them, but the possibility of perforation should be noted. Early surgical treatment should be performed for irretrievable intussusception, complete intestinal obstruction, intestinal perforation area ≥ 1 cm, malignant polyps, and failure of conservative treatment. PJS patients < 10 years old may undergo noninvasive examinations such as abdominal ultrasound, CT, capsule endoscopy, gastrointestinal contrast, or magnetic resonance imaging to assess the burden and diameter of their gastrointestinal polyps. If polyps < 10 mm are found, resection can be attempted for a smaller quantity of polyps, but if they cannot be removed, a biopsy will be required to determine the pathology of the polyps and estimate the next follow-up time, and the growth rate of the polyps needs to be closely monitored. For giant polyps, multiple endoscopic resections should be carried out, but for diffuse distributions of polyps, the complete resection of all polyps in the entire intestinal canal should not be excessively pursued in order to prevent short bowel syndrome, and the patients should be monitored every 1 to 2 years (Figure 2).

CONCLUSION

The development of gastrointestinal symptoms in PJS patients is closely related to the age of the appearance of mucocutaneous pigmentation and polyp burden and diameter. The later the age of mucocutaneous pigmentation, the more severe the gastrointestinal symptoms, and patients receive more frequent operations and hospitalization. As PJS polyps have a high risk of intussusception and carcinogenesis, patients aged ≥ 10 years should undergo a small intestinal enteroscopy every 1–2 years.

Endoscopic treatment has a good safety profile and can have significant beneficial effects for PJS patients, and timely endoscopic treatment can reduce the risk of the intussusception and carcinogenesis of polyps.

ARTICLE HIGHLIGHTS

Research background

Peutz-Jeghers syndrome (PJS) is a clinically rare autosomal dominant inherited disease with pigmented spots on the lips and mucous membranes and extremities, scattered gastrointestinal polyps, and susceptibility to tumors as clinical manifestations. Effective preventive and curative methods are still lacking.

Research motivation

Here we summarize out experience with 566 Chinese patients with PJS from a Chinese medical center our experience with 566 Chinese patients with PJS from a Chinese medical center with regard to the clinical features, diagnosis, and treatment, in order to promote the clinical understanding of PJS and improve its clinical diagnosis and treatment.

Research objectives

To explore the clinical features, diagnosis, and treatment of PJS in Chinese patients.

Research methods

The clinical data of 566 PJS cases admitted to the Air Force Medical Center from January 1994 to October 2022 was retrospectively analyzed, including age, gender, ethnicity, family history, first treatment age, time and sequence of appearance of mucocutaneous pigmentation, polyp distribution, polyp quantity and diameter, frequency of hospitalization, frequency of surgical operations, *etc.*

Research results

Of all the patients included, 55.3% were male and 44.7% were female. Median time to the appearance of mucocutaneous pigmentation was 2 years, and median time from the appearance of mucocutaneous pigmentation to the occurrence of abdominal symptoms was 10 years. The vast majority (92.2%) of patients underwent small bowel endoscopy and treatment, with 2.3% having serious complications. There was a statistically significant difference in the number of enteroscopies between patients with and without canceration (P = 0.004, Z = -2.882); 71.2% of patients underwent surgical operation, 75.6% of patients underwent surgical operation before the age of 35 years, and there was a statistically significant difference in the frequency of surgical operations between patients with and without cancer (P = 0.000, Z = -5.127). At 40 years of age, the cumulative risk of intussusception in PJS was approximately 72.0%, and at 50 years, the cumulative risk of intussusception in PJS was approximately 89.6%. At 50 years of age, the cumulative risk of cancer in PJS was approximately 49.3%, and at 60 years of age, the cumulative risk of cancer in PJS was approximately 71.7%.

Research conclusions

The risk of intussusception and cancer of PJS polyps increases with age. PJS patients ≥ 10 years old should undergo annual enteroscopy. Endoscopic treatment has a good safety profile and can reduce the occurrence of polyp intussusception and cancer. Surgery should be conducted to protect the gastrointestinal system by removing polyps.

Research perspectives

The clinical data of 566 PJS cases diagnosed and treated in a Chinese medical center were retrospectively analyzed, and the clinical characteristics and diagnosis and treatment process of Chinese PJS are summarized.

FOOTNOTES

Author contributions: Xu ZX, Jiang LX, and Chen YR contributed equally to this study; Gu GL designed the research; Xu ZX, Jiang LX, Chen YR, Zhang YH, Zhang Z, Yu PF, Dong ZW, and Yang HR collected and analyzed the clinical data; Xu ZX, Jiang LX, and Chen YR wrote the manuscript; Gu GL and Dong ZW revised the manuscript.

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ORIGINAL ARTICLE

Observational Study

Intraprocedural gastric juice analysis as compared to rapid urease test for real-time detection of *Helicobacter pylori*

Riccardo Vasapolli, Florent Ailloud, Sebastian Suerbaum, Jens Neumann, Nadine Koch, Lukas Macke, Jörg Schirra, Julia Mayerle, Peter Malfertheiner, Christian Schulz

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Abstract

BACKGROUND

Endofaster is an innovative technology that can be combined with upper gastrointestinal endoscopy (UGE) to perform gastric juice analysis and real-time detection of *Helicobacter pylori* (*H. pylori*).

AIM

To assess the diagnostic performance of this technology and its impact on the management of *H. pylori* in the real-life clinical setting.

METHODS

Patients undergoing routine UGE were prospectively recruited. Biopsies were taken to assess gastric histology according to the updated Sydney system and for rapid urease test (RUT). Gastric juice sampling and analysis was performed using the Endofaster, and the diagnosis of *H. pylori* was based on real-time ammonium measurements. Histological detection of *H. pylori* served as the diagnostic gold standard for comparing Endofaster-based *H. pylori* diagnosis with RUT-based *H.*

pylori detection.

RESULTS

A total of 198 patients were prospectively enrolled in an *H. pylori* diagnostic study by Endofaster-based gastric juice analysis (EGJA) during the UGE. Biopsies for RUT and histological assessment were performed on 161 patients (82 men and 79 women, mean age 54.8 ± 19.2 years). *H. pylori* infection was detected by histology in 47 (29.2%) patients. Overall, the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value (NPV) for *H. pylori* diagnosis by EGJA were 91.5%, 93.0%, 92.6%, 84.3%, and 96.4%, respectively. In patients on treatment with proton pump inhibitors, diagnostic sensitivity was reduced by 27.3%, while specificity and NPV were unaffected. EGJA and RUT were comparable in diagnostic performance and highly concordant in *H. pylori* detection (κ-value = 0.85).

CONCLUSION

Endofaster allows for rapid and highly accurate detection of *H. pylori* during gastroscopy. This may guide taking additional biopsies for antibiotic susceptibility testing during the same procedure and then selecting an individually tailored eradication regimen.

Key Words: *Helicobacter pylori* diagnostic; Chronic gastritis; Gastric juice; Endofaster; Rapid urease test; Antimicrobial susceptibility testing

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Core Tip: Diagnosis of *Helicobacter pylori* (*H. pylori*) infection can be rapidly achieved within the framework of gastroscopy by rapid urease test (RUT) or by gastric juice analysis with Endofaster. In this prospective observational study, we compared the accuracy of these two methods. Gastric juice analysis with Endofaster could reliably detect *H. pylori* with high accuracy, showing a diagnostic performance comparable to that of RUT and a major advantage of an immediate result. Intraprocedural *H. pylori* detection (or exclusion) is crucial to optimize the diagnostic approach and improve the management of infection. The diagnosis of Endofaster may guide additional sampling for antibiotic susceptibility testing in positive patients or avoid unnecessary biopsies in negative patients.

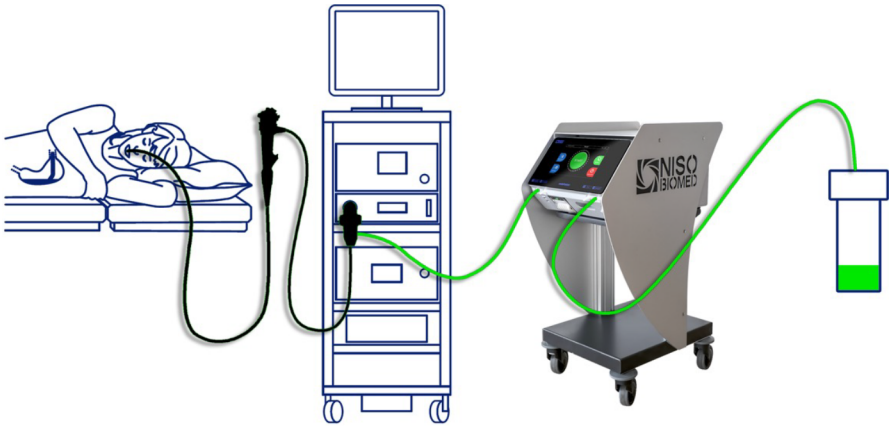
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INTRODUCTION

Helicobacter pylori (*H. pylori*) infects nearly half of the world's population, with variable prevalence rates ranging from 20%-30% in Western countries to > 70% in Africa[1]. *H. pylori* infection causes chronic active gastritis and may lead to severe complications including gastroduodenal ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma[2]. The diagnosis of active *H. pylori* infection is achieved by non-invasive tests such as the urea breath test (UBT) and stool antigen tests (SAT), as well as invasive methods based on endoscopy and gastric biopsies for histological assessment, rapid urease test (RUT), culture and molecular tests.

Current guidelines recommend testing for *H. pylori* in all patients undergoing upper gastrointestinal endoscopy (UGE)[3]. The Endofaster has been introduced as new diagnostic device, which consents the detection of *H. pylori* by performing biochemical analysis of gastric juice aspirated during gastroscopy. Previous validation studies have shown that this device has high accuracy for *H. pylori* detection and reported diagnostic values similar to those of UBT and histology[4,5]. The diagnostic performance of the Endofaster has not been compared with that of the RUT, which shares a similar characteristic in terms of providing results in a short-term temporal context through endoscopic examination. This allows for therapeutic management immediately after the diagnostic procedure.

The aim of this prospective study was to validate the diagnostic performance of the Endofaster for *H. pylori* detection in patients who underwent UGE compared to conventional RUT.



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Figure 1 Schematic diagram of Endofaster device's montage.

MATERIALS AND METHODS

Study population

Consecutive patients undergoing routine UGE to investigate dyspepsia or other alarming symptoms (weight loss, anemia, vomiting, abdominal pain, or dysphagia) were prospectively recruited at the Ludwig Maximilians University Hospital in Munich from January to June 2022.

Subjects were recruited within the ERANET Bavaria and Helicopredict projects (German clinical trials register, DRKS-ID: DRKS00028629), large-scale prospective studies focused on studying different aspects of *H. pylori* infection, including improving the diagnosis and management of *H. pylori*, determining local antibiotic resistance spectrum, with the aim of developing a genotypic resistance testing database for predicting antibiotic susceptibility and evaluating the impact of the microbiome of the upper gastrointestinal tract on gastric carcinogenesis.

The study was approved by the local ethics committee and government authorities and was conducted in accordance with current Good Clinical Practice guidelines and the Declaration of Helsinki [6]. All recruited subjects provided written informed consent for participation. Previous gastric surgery and intake of anticoagulants or any antibiotic therapy within 4 wk prior to endoscopy were exclusion criteria. Regular use of proton pump inhibitors (PPI) or previous *H. pylori* eradication therapy did not represent exclusion criteria, but were recorded in detail. Only patients not taking a PPI or *H. pylori* treatment-naïve patients were considered to meet the desired minimum sample size.

Endoscopic procedure and histological assessment

Enrolled patients underwent a diagnostic UGE using standard video gastroscopes (GIF-HQ190, Olympus, Tokyo, Japan). All examinations were performed with sedation using Propofol and/or Midazolam. An analysis of gastric juice was performed at the beginning of the UGE by Endofaster. Special attention was paid during intubation: The stomach was handled first and no fluid was allowed to be sucked during passage through the oral cavity or esophagus. In order to avoid possible dilution of gastric juice prior to collection the administration of endoscopic premedications (*i.e.* dimethicone, N-acetylcysteine, pronase *etc.*) before endoscopy were not allowed. Furthermore, washing with water and cleaning the endoscopic lens were avoided until sampling was completed. After endoscopic assessment of the mucosa, gastric biopsies were obtained. Two biopsies - one from antrum and one from corpus (both from the greater curvature) - were taken for the RUT (Pronto Dry® New, Medical Instruments Corporation, Herford, Germany). RUT was performed according to the manufacturer's instructions and assessed for positive response during gastroscopy and 1 h after biopsy sampling. The inspection time taken to perform the diagnostic UGE (excluding the time spent on gastric juice aspiration and on biopsy sampling) and the time it took until first detection of *H. pylori* positivity by RUT were recorded. Further biopsies (2 from antrum, 1 from angulus and 2 from corpus) were subjected to routine histology according to the updated Sydney system[7] and current guidelines[3]. In each biopsy sampling set the following stainings were performed: Hematoxylin and eosin, periodic acid-Schiff and a *H. pylori* specific staining (modified Giemsa staining).

Endofaster analysis

Real-time gastric juice analysis was performed using an Endofaster 21-42 (NISO Biomed, Turin, Italy), which is interposed between the endoscope and the suction system (Figure 1). This innovative device analyzes the first 3.3 mL of gastric juice aspirated at the beginning of the UGE. The Endofaster provides information regarding gastric pH based on hydrogen ion concentration and *H. pylori* detection based on

the measurement of ammonium derived from bacterial urease activity within 60-90 s[4,5]. Considering that approximately 10-20 s (max 30 s) are needed to aspirate the gastric juice through the scope a final *H. pylori* diagnosis is provided within the first 2 min from the beginning of the endoscopic procedure. Except for the time spent on the initial gastric juice collection no additional time is required for Endofaster use during the endoscopic procedure. In line with previous studies, we used a cut-off value of > 62 ppm/mL to indicate the presence of *H. pylori*[8].

Statistical analyses

Descriptive statistical analysis was performed using IBM SPSS Statistics 23.0.0 (IBM Corporation, New York, NY, United States). Numerical variables were expressed as mean ± SD. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were calculated for both Endofaster and RUT using histology as the gold standard. The concordance between Endofaster and RUT results was assessed by using Cohen's κ-value. The McNemar test was used to compare sensitivities and specificities between the two tests.

Sample size estimation was based on a 95%CI and the calculation methods of Buderer *et al*[9] were applied using following formula:

$$n_{Se} = \frac{Z^2_{\frac{\alpha}{2}} \hat{Se}\,(1 - \hat{Se})}{d^2 \times Prev}$$

$$n_{Sp} = \frac{Z^2_{\frac{\alpha}{2}} \hat{Sp}\,(1 - \hat{Sp})}{d^2 \times (1 - Prev)}$$

Where Z is the normal distribution value set to 1.96, corresponding to the 95%CI, and d is the maximum acceptable width of the 95%CI, set at 10%. Based on a previous study, Endofaster had a sensitivity (*Se*) of 97.1% and a specificity (*Sp*) of 89.7% for *H. pylori* detection[5]. Recently, the prevalence of *H. pylori* infection (*Prev*) in Germany was estimated to be 35.3% (95%CI: 31.2-39.4)[1]. As a result, using the criteria listed above, this study required a minimum of 31 *H. pylori*-positive patients (n_{Se}) and 55 *H. pylori*-negative patients (n_{Sp}), resulting in a minimum total sample size of 86 subjects. Patients with PPI use or prior *H. pylori* eradication therapy were not considered to achieve the minimum sample size required. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Characteristics of the study cohort

A total of 198 patients undergoing elective UGE were enrolled. Of these, 182 underwent gastric juice analysis with the Endofaster. After excluding patients who reported antibiotic intake within the last 4 wk (n = 10, 5.2%), patients who could not undergo biopsy due to anticoagulation therapy (n = 8, 4.1%) and patients with insufficient volume of aspirated gastric juice for Endofaster analysis (n = 13, 6.7%) a total of 161 patients (male: 82, female: 79, mean age 54.8 ± 19.2 years) were included in the analysis. 67 (41.6%) patients were on ongoing PPI therapy and 94 patients (58.4%) did not report any PPI therapy. The demographic, endoscopic, and histopathological characteristics of the study cohort are shown in Table 1. A flow chart of the study's recruitment is shown in Figure 2.

Diagnostic performance of Endofaster and RUT for H. pylori detection

The average duration of the diagnostic UGE was 8.5 min. *H. pylori* infection was diagnosed in 47 (29.2%) patients on histopathology. Endofaster results were positive in 51 patients (31.6%), while RUT was positive in 45 (28.0%) cases. A positive RUT reaction was detected during endoscopy in 37 subjects (78.7%), with a mean positive reaction time of 16.4 min. The overall diagnostic performances of Endofaster and RUT for *H. pylori* detection as compared to histology (gold standard) are shown in Table 2. Sensitivity, specificity, accuracy, PPV and NPV were 91.5%, 93.0%, 92.6%, 84.3% and 96.4% for Endofaster, and 93.6%, 99.1%, 97.5%, 97.8% and 97.4% for RUT, respectively. No significant differences were observed in the diagnostic performances of the Endofaster and the RUT (P > 0.05). This was confirmed by an almost perfect agreement of *H. pylori* detection between the two tests (κ-value = 0.85).

Both Endofaster and RUT showed excellent diagnostic performances when considering only patients without ongoing PPI therapy (n = 94). In this subgroup, 37 (39.4%) subjects were histopathologically diagnosed as positive for *H. pylori*.

Among patients treated with PPI (n = 67), the presence of *H. pylori* was detected by histology in 10 subjects (14.9%). In this subgroup, a reduction in sensitivity, PPV and accuracy was observed for both Endofaster and RUT, whereas specificity and NPV remained almost unchanged (Table 2). Again, in the subgroup analysis, there were no significant differences in diagnostic performances between Endofaster and RUT (P > 0.05).

Table 1 Demographic, endoscopic and histopathological characteristics of the patients included in the study, *n* (%)

Characteristics	Value
Overall	161
Male	82 (50.9)
Female	79 (49.1)
Age, mean ± SD (range) yr	54.8 ± 19.2 (19-90)
H. pylori positive	47 (29.2)
H. pylori negative	114 (70.8)
Patients without PPI therapy	94 (58.4)
Male	46 (48.9)
Female	48 (51.1)
Age, mean ± SD (range) yr	50.3 ± 19.2 (19-86)
H. pylori positive	37 (39.4)
H. pylori negative	57 (60.6)
Patients with PPI therapy	67 (41.6)
Male	36 (53.7)
Female	31 (46.3)
Age, mean ± SD (range) yr	58.9 ± 19.2 (23-90)
H. pylori positive	10 (14.9)
H. pylori negative	57 (85.1)
Endoscopic and histopathological findings[1]	
Normal	13 (8.1)
Gastroesophageal reflux disease	26 (16.1)
Chronic gastritis	84 (52.2)
Erosive gastritis	32 (19.9)
Gastric ulcer	5 (3.1)
Duodenal ulcer	3 (1.9)
Gastritis with low-grade PL	36 (22.4)
Gastritis with high-grade PL	4 (2.5)
Others[2]	6 (3.7)

[1]Different conditions may coexist. Precancerous lesions include gastric atrophy and/or intestinal metaplasia, assessed according to the Operative Link on Gastritis Assessment (OLGA) and Operative Link on Gastric Intestinal Metaplasia systems (low grade if OLGA/IM < 3, high grade if OLGA/IM ≥ 3)[23, 24].

[2]"Others" include patients with gastric cancer (*n* = 1), gastric lymphoma (*n* = 1), Barrett's esophagus (*n* = 2), gastric hyperplastic polyps (*n* = 1) and gastric neuroendocrine tumors (*n* = 1).

H. pylori: *Helicobacter pylori*; PPI: Proton pump inhibitors.

DISCUSSION

Several diagnostic methods are performed on biopsies obtained during the UGE to detect *H. pylori* with high accuracy. They are highly accurate, but have the limitation to delay even a few days in providing diagnostic results, thus not allowing an immediate therapeutic decision. RUT is the only exception in clinical practice that allows relatively rapid detection of *H. pylori*, usually within 1 h after UGE[10-12].

Here, we report on the diagnostic performance of Endofaster-based gastric juice analysis (EGJA), an innovative technology that allows intraprocedural *H. pylori* detection compared to RUT. We found that the high accuracy (> 90%) of EGJA was comparable to that of RUT for *H. pylori* detection, confirming previous reports of the high accuracy of EGJA compared to histology[4,5,8,13]. A previous prospective study of EGJA in 182 patients determined the sensitivity, specificity and accuracy of *H. pylori* to be 97.1%, 89.7% and 92.6%, respectively, compared to histology being used as the gold standard as well as

Table 2 Diagnostic performance of the Endofaster® and rapid urease test (ProntoDry®) for the diagnosis of *Helicobacter pylori* infection in the study cohort, % (95%CI)

	Endofaster			Rapid urease test		
	Overall	No PPI	Ongoing PPI therapy	Overall	No PPI	Ongoing PPI therapy
Sensitivity	91.5 (79.6-97.6)	97.3 (85.8-99.9)	70.0 (34.8-93.3)	93.6 (82.5-98.7)	97.3 (85.8-99.9)	80.0 (44.4-97.5)
Specificity	93.0 (84.6-96.9)	96.5 (87.9-99.6)	89.5 (78.5-96.0)	99.1 (95.2-100)	100 (93.7-100)	98.3 (90.6-100)
PPV	84.3 (73.3-91.3)	94.7 (82.2-98.6)	53.9 (33.1-73.4)	97.8 (86.2-99.7)	100 (-)	88.9 (52.8-98.3)
NPV	96.4 (91.2-98.6)	98.2 (88.8-99.7)	94.4 (86.8-97.8)	97.4 (92.7-99.1)	98.3 (89.2-99.8)	96.6 (89.0-99.0)
Accuracy	92.6 (87.3-96.1)	96.8 (91.0-99.3)	86.6 (76.0-93.7)	97.5 (93.8-99.3)	98.9 (94.2-100)	95.5 (87.5-99.1)

Overall (n = 161), in patients with ongoing proton pump inhibitors (PPI) therapy (n = 67) and in patients without PPI therapy (n = 94). Values are shown as percentages with 95%CI. PPI: Proton pump inhibitor; PPV: Positive predictive value; NPV: Negative predictive value.

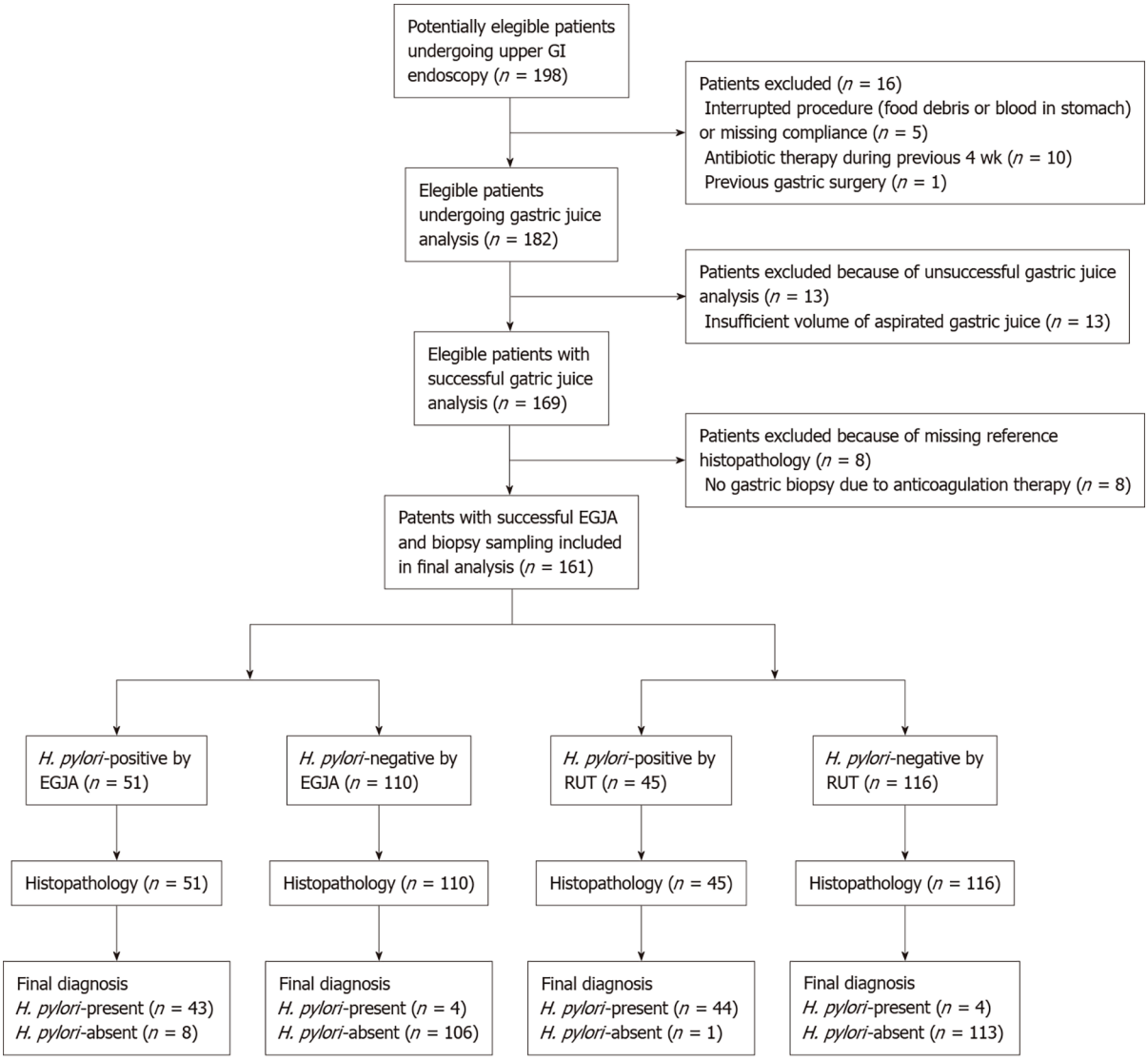
UBT, which was used for reclassification of *H. pylori* status in case of discordance between EGJA and histology results[5]. A multicenter study of 525 consecutive patients reported an overall sensitivity, specificity and accuracy of 87%, 84% and 85%, respectively, when compared to histology[8].

We have observed impaired diagnostic sensitivity in patients with PPI in EGJA and RUT, which is a common phenomenon for all tests, including non-invasive tests[3,14]. In the context of PPI intake, only histology remains highly sensitive when gastric biopsies are taken from the proximal stomach[15,16]. This is related to the PPI-induced shift from antrum-predominant to corpus-predominant gastritis. We found that 3 out of 4 false negatives and 6 out of 8 false positives (75%) in EGJA were registered in patients on PPI therapy. Two of the three false negatives (66%) diagnosed by the RUT were PPI users. EGJA and RUT rely on the same principles related to ammonium concentration and *H. pylori* urease activity. Therefore, both tests are influenced by the reduction of bacterial load by PPI, which may lead to false negative results. Furthermore, an elevated pH in the stomach environment may lead to an overgrowth of other non-*H. pylori* bacteria with urease activity[17]. Several different urease-positive bacterial strains, such as *Staphylococcus capitis subsp. urealyticus* and *Streptococcus salivarium*, have been isolated in gastric juice and mucosal samples from patients with gastric hypochloridria[18]. The higher abundance of these strains may interfere with urea metabolism and explain the increased number of false-positive cases among patients on PPI therapy. It is necessary to analyze the gastric microbiota and functionality profiles of PPI patients in order to further address this interesting topic. In our study, the low prevalence of *H. pylori*-infected subjects (only 14.9%) within the group of patients on PPI therapy is a limitation because of an underpowered statistical analysis. Using histology as the gold standard for *H. pylori*-diagnosis in a cohort with relatively low-prevalence of *H. pylori* may represent a further limitation of this study. Histopathological diagnosis of *H. pylori* may suffer from potential sampling error due to the patchy distribution of the bacterium[19]. However, by using the updated Sydney system based on biopsies from 5 different sites and applying different staining methods for *H. pylori* detection the accuracy of *H. pylori*-diagnosis by histology is not inferior to any non-invasive test (13C-UBT/SAT). In support for the validity of histology as gold standard for *H. pylori* detection, we found also no indirect signs of *H. pylori*-gastritis (*i.e.* neutrophils infiltration in the gastric mucosa) in the absence of *H. pylori*.

EGJA has the advantage of obtaining more rapid diagnostic results when performing endoscopy compared to RUT. During endoscopy (within a time period of approximately 10 min), a positive signal in the RUT for the presence of *H. pylori* was recorded in 78.7% of those producing *H. pylori* positivity at the end of the reading time in our study, consistent with the time interval of response reported in previous validation studies[12,20], whereas EGJA resulted in the diagnosis of *H. pylori* within 2 min after starting with UGE. The intraprocedural detection of *H. pylori* infection combined with measurement of gastric pH can guide the endoscopist on the most appropriate approach to complete the diagnostic assessment, *i.e.*, whether or not to carry out additional biopsies for gastritis severity staging and antibiotic susceptibility testing (AST). This has become an absolute requirement for the selection of the eradication regimen due to the high antibiotic resistance rates of clarithromycin, metronidazole and fluoroquinolones[21]. Real-time detection of *H. pylori* suggests carrying out additional biopsies for AST during UGE and selecting an *H. pylori* eradication regimen accordingly.

Such a strategy would have a substantial impact on cost-effectiveness by reducing the duration of the procedure and lowering costs due to histological or microbiological analysis of negative gastric biopsies, an aspect that has been previously addressed by others[8].

Future studies will explore the possibility of combining EGJA with *in situ* molecular genetic antibiotic resistance testing. Promising data in this field were revealed by a recent meta-analysis of four studies that evaluated gastric juice-based genotypic detection of *H. pylori* antibiotic resistance to clarithromycin compared to standard culture-based methods[22].



DOI: 10.3748/wjg.v29.i10.1638 **Copyright** ©The Author(s) 2023.

Figure 2 Flowchart according to Standards for Reporting Diagnostic Accuracy Studies guidelines of patient inclusion in the study and analysis. EGJA: Endofaster-based gastric juice analysis; *H. pylori*: *Helicobacter pylori*; RUT: Rapid urease test.

CONCLUSION

In conclusion, Endofaster's gastric juice analysis is a highly accurate method for the diagnosis of *H. pylori* infection, comparable to RUT. EGJA-based *H. pylori* diagnosis has an advantage in terms of on-site immediacy of diagnosis. In patients on PPI therapy, sensitivity is reduced, but NPV and specificity are not affected. Real-time detection of *H. pylori* along with the determination of gastric pH during endoscopy adds important information on the need for additional biopsies for more detailed histological assessment and antibiotic susceptibility testing.

ARTICLE HIGHLIGHTS

Research background

Diagnosis of *Helicobacter pylori* (*H. pylori*) infection can be rapidly achieved within the framework of gastroscopy by rapid urease test (RUT) or by gastric juice analysis with Endofaster.

Research motivation

The diagnostic performance of the Endofaster has not been compared with that of the RUT, which shares a similar characteristic in terms of providing results in a short-term temporal context through endoscopic examination.

Research objectives

The objective of this prospective study was to validate the diagnostic performance of the Endofaster for *H. pylori* detection in patients who underwent gastroscopy compared to the diagnostic accuracy of a standard RUT.

Research methods

Patients undergoing routine upper gastrointestinal endoscopy were prospectively recruited. Biopsies were taken to assess gastric histology according to the updated Sydney system and for RUT. Gastric juice sampling and analysis was performed using the Endofaster, and the diagnosis of *H. pylori* was based on real-time ammonium measurements. Histological detection of *H. pylori* served as the diagnostic gold standard for comparing Endofaster-based *H. pylori* diagnosis with RUT-based *H. pylori* detection.

Research results

Gastric juice analysis with Endofaster could reliably detect *H. pylori* with an overall sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of 91.5%, 93.0%, 92.6%, 84.3%, and 96.4%, respectively. Gastric juice analysis with Endofaster and RUT were comparable in diagnostic performance and highly concordant in *H. pylori* detection (κ-value = 0.85).

Research conclusions

Endofaster's gastric juice analysis is a highly accurate method for the diagnosis of *H. pylori* infection, comparable to RUT. EGJA-based *H. pylori* diagnosis has an advantage in terms of on-site immediacy of diagnosis.

Research perspectives

Intraprocedural diagnosis of *H. pylori*-infection by Endofaster may guide additional sampling for antibiotic susceptibility testing in positive patients or avoid unnecessary biopsies in negative patients.

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FOOTNOTES

Author contributions: Vasapolli R, Schulz C, and Malfertheiner P designed the study, interpreted the data and drafted the manuscript; Vasapolli R, Suerbaum S, Schirra J, Mayerle J, Schulz C, and Malfertheiner P obtained funding and supervised the study procedures; Vasapolli R recruited suitable subjects, performed endoscopy, and collected samples; Vasapolli R, Macke L, Ailloud F, and Koch N registered data and performed statistical analyses; Neumann J performed the histological analysis; All authors read and approved the final version of the manuscript.

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Informed consent statement: Written informed consent was obtained from the participants.

Conflict-of-interest statement: The authors have nothing to disclose.

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STROBE statement: The authors have read the STROBE statement, and the manuscript was prepared and revised according to the STROBE statement.

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LETTER TO THE EDITOR

Reporting the cases of alcohol-associated hepatitis using the National Inpatient Sample data

Natalie Marlowe, Wei-Qi Lin, Suthat Liangpunsakul

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Abstract

The letter is to respond to the recent publication "Trends in hospitalization for alcoholic hepatitis from 2011 to 2017: A USA nationwide study" (*World J Gastroenterol* 2022; 28: 5036-5046). We noticed a significant difference in the total numbers of reported hospitalized alcohol-associated hepatitis (AH) patients between this publication and our publication on *Alcohol Clin Exp Res* (2022; 46: 1472-1481). We believe the number of "AH-related hospitalizations" inflated by the inclusion of patients with non-AH forms of alcohol-associated liver disease.

Key Words: Hospitalization; Alcoholic hepatitis; Alcohol-associated liver disease

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Core Tip: We analyzed the most recent National Inpatient Sample data from 2015-2019 using International Classification of Diseases-10 codes and found an increase in alcohol-associated hepatitis (AH) cases from 110135 to 136620 in 2015 and 2019, respectively. The total numbers of reported AH patients in the retrospective study entitled "Trends in hospitalization for alcoholic hepatitis from 2011 to 2017: A USA nationwide study", we believe, included patients with non-AH forms of alcohol-associated liver disease.

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TO THE EDITOR

We read with great interest the retrospective study entitled "Trends in hospitalization for alcoholic hepatitis from 2011 to 2017: A USA nationwide study" by Wakil *et al*[1]. In this study the authors examined inpatient admission trends for alcohol-associated hepatitis (AH), using the National Inpatient Sample (NIS) database data from 2011 to 2017. The study population were those with aged ≥ 21 years who were hospitalized with either a primary or secondary diagnosis of AH identified by the International Classification of Diseases (ICD)-9 and its corresponding ICD-10 codes.

The authors reported that AH-related hospitalization demonstrated a significant increase from 281506 in 2011 to 324050 hospitalizations in 2017 with an overall increase in the financial burden and cost. We agree with the authors' opinion that AH-related hospitalizations are on the rise, and that they are associated with escalating healthcare costs and utilization. In fact, our recent paper published in *Alcohol Clin Exp Res*, 2022, came to the same conclusions when we analyzed the most recent NIS data for AH hospital discharges from 2015-2019 using a similar methodology[2]. However, we noticed a significant difference in the total numbers of reported AH patients between these studies. In our study, we reported an increase in total hospitalized AH cases from 110135 to 136620 in 2015 and 2019, respectively [2], which was consistent with another recent paper by Ali *et al*[3] in *Annals of Gastroenterology*, 2022. The numbers from both Ali *et al*[3] and our study were much smaller than the numbers quoted by Wakil *et al* [1]. The difference is explained by the ICD codes used in the studies. We included only patients hospitalized with or without cirrhosis under ICD-9 571.1 (AH) and ICD-10 K70.1 (AH with/without ascites). In contrast, Wakil *et al*[1] included patients admitted with AH, alcoholic fatty liver disease, and alcohol-associated cirrhosis [an advanced chronic form of associated liver disease (ALD)]. In our opinion, the number of "AH-related hospitalizations" reported by this study is inflated by the inclusion of patients with non-AH forms of ALD.

Alcohol use and ALD are global public health issues associated with high morbidity and mortality [4]. Retrospective studies investigating AH hospitalization trends and associated healthcare costs should be interpreted with caution because the results may be influenced significantly by the ICD codes used to identify AH patients. Reporting using the most appropriate codes is the best use of these large national databases.

FOOTNOTES

Author contributions: Marlowe N and Lin WQ drafted the manuscript; Liangpunsakul S criticized and edited the manuscript.

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REVIEW

Microbiome-liver crosstalk: A multihit therapeutic target for liver disease

Jorum Kirundi, Sheida Moghadamrad, Camilla Urbaniak

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Abstract

Liver disease has become a leading cause of death, particularly in the West, where it is attributed to more than two million deaths annually. The correlation between gut microbiota and liver disease is still not fully understood. However, it is well known that gut dysbiosis accompanied by a leaky gut causes an increase in lipopolysaccharides in circulation, which in turn evoke massive hepatic inflammation promoting liver cirrhosis. Microbial dysbiosis also leads to poor bile acid metabolism and low short-chain fatty acids, all of which exacerbate the inflammatory response of liver cells. Gut microbial homeostasis is maintained through intricate processes that ensure that commensal microbes adapt to the low oxygen potential of the gut and that they rapidly occupy all the intestinal niches, thus outcompeting any potential pathogens for available nutrients. The crosstalk between the gut microbiota and its metabolites also guarantee an intact gut barrier. These processes that protect against destabilization of gut microbes by potential entry of pathogenic bacteria are collectively called colonization resistance and are equally essential for liver health. In this review, we shall investigate how the mechanisms of colonization resistance influence the liver in health and disease and the microbial-liver crosstalk potential as therapeutic target areas.

Key Words: Microbiome; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver disease; Microbiome-host crosstalk; Gut homeostasis; Microbial metabolites

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Core Tip: The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms of how the microbiome contributes to liver disease are unknown, there is strong evidence that the translocation of various metabolites across the mucosal barrier plays a strong role, which is precipitated by dysbiotic gut microbiota. Considering the importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. This review will explore how the mechanisms of colonization resistance influence the liver in health and disease and finally examine potential therapeutic targets in the gut-liver axis.

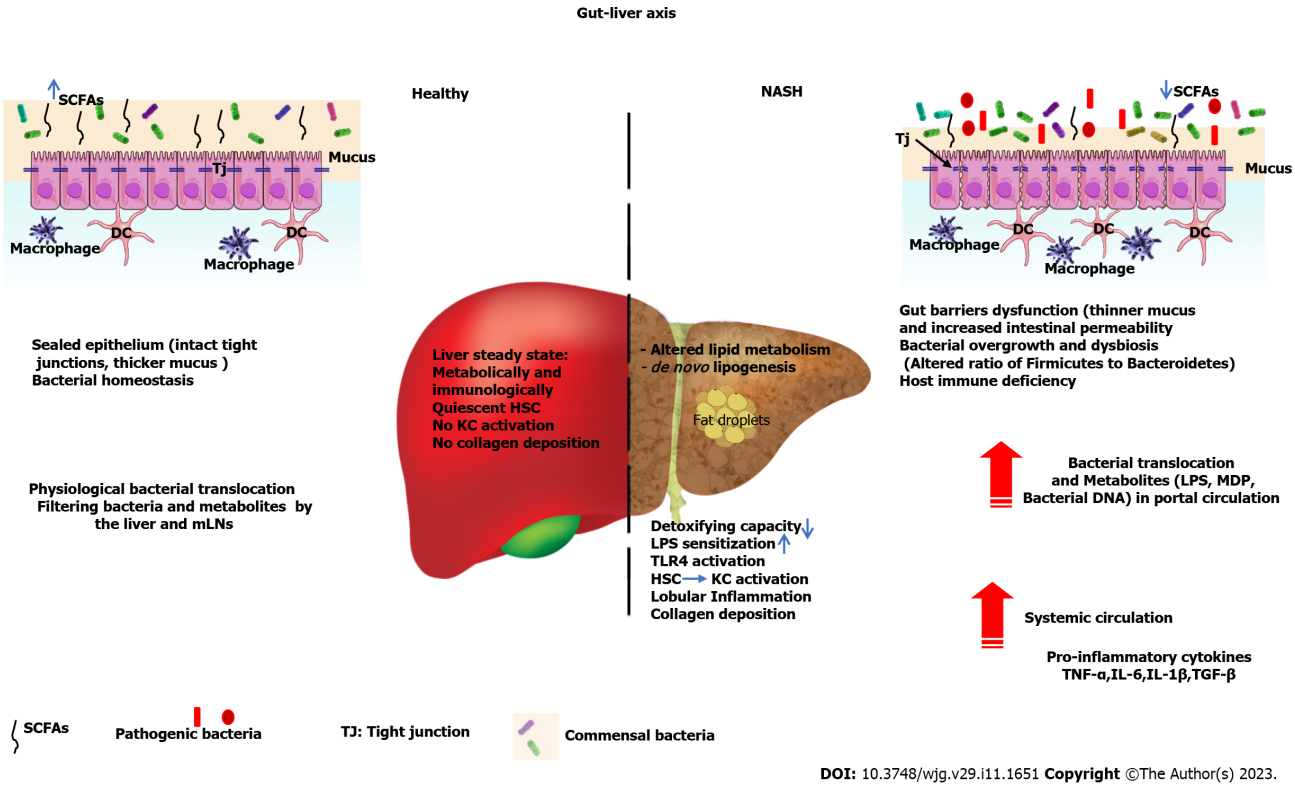
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INTRODUCTION

A healthy gut microbiota plays a significant role in maintaining a homeostatic gut environment. One such role is colonization resistance, which is defined as the microbial capacity to resist invasion of exogenous microorganisms (for example, pathogens) and/or prevent uncontrolled overgrowth of endogenous microbes (for example, pathobionts). For gut homeostasis to be achieved, microbial alpha diversity must remain high, gut mucosal integrity must be maintained, and tolerance to the billions of microbial immunogens present in the gut must be established. This is all achieved through intricate microbe-to-microbe and microbe-to-host interactions mediated by microbial metabolites, such as short-chain fatty acids, or microbial cell wall components, such as lipopolysaccharides, lipoteichoic acid, peptidoglycans and flagellin. Homeostasis is also achieved through the production of antimicrobial peptides, resource and oxygen competition, host immunomodulation, and conjugation of bile acids. The mechanisms by which these inter/intramicrobial interactions mediate colonization resistance or how their perturbation leads to disease have not yet been fully elucidated. However, it is known that an imbalance in microbial composition, otherwise known as dysbiosis, which may arise from dietary changes, ingestion of exogenous toxins such as antibiotics or xenobiotics, or through infections that suppress the immune system, has serious and sometimes long-term clinical implications. Diseases such as diabetes, obesity, atherosclerosis, and liver disease are associated with dysbiosis and the translocation of gut microbial products into circulation. As the liver is the first organ to be exposed to the gut bacterial products and digested food delivered through the portal vein, any leakage of microbial products into circulation will lead to hepatocellular immune activation, thereby promoting systemic and hepatic inflammation, which may lead to liver disease[1]. An understanding of the mechanisms involved in colonization resistance and its influencing factors is therefore crucial to establish their link to the etiology of liver disease as well as to identify possible hit points along the gut-liver axis that can be utilized as therapeutic targets for liver disease[2]. This review explores some of the mechanisms of colonization resistance and their importance to the etiology of the different stages of nonalcoholic fatty liver disease (NAFLD) from simple steatosis to liver inflammation, as well as alcohol-associated liver disease (ALD), and highlights potential entry points that may be used as therapeutic targets for liver disease. A summary of the interplay between the microbiome, liver, immune system, and metabolome is presented in Figure 1.

GUT MICROBIAL EUBIOSIS

The gut microbiome starts taking shape at birth, where it is initially influenced by the mode of delivery. Vaginally born babies will have a gut microbial composition very close to the maternal vaginal microbiota, while the caesarian born will adopt mainly the skin microbiota[3]. Mammals have five phyla that predominate the gut: Firmicutes (*e.g.*, *Lactobacillus, Clostridium, Ruminococcus, Eubacterium, Fecalibacterium and Roseburia*), Actinobacteria (with *Bifidobacterium* as one of its most important members), Bacteroidetes (*e.g.*, *Bacteroides, Prevotella, and Xylanibacter*), Proteobacteria (*e.g.*, *Escherichia* and *Desulfovibrio*) and Verrucomicrobia (*e.g.*, *Akkermansia*)[4]. The earliest colonizers are mainly facultative aerobes of the phyla Firmicutes and Actinobacteria, which play a significant role in lowering the gut's oxygen level to allow for the colonization of obligate anaerobes. These aerotolerant microbes reside in the upper gut, where they continue to reduce the amount of oxygen in the gut for life. *Escherichia coli* and *Enterococcus faecalis* are the most abundant in the oxygen-high neonatal gut, and they rapidly expand in the early phase, leading to a gradual depression of oxygen levels and allowing growth of the facultative



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Figure 1 During development of nonalcoholic steatohepatitis, several immunological and metabolic pathways intersect, thus promoting progression of liver injury and nonalcoholic steatohepatitis. In healthy conditions, gut-liver axis homeostasis is guaranteed by intact intestinal epithelium barriers and proper liver-host immune functions that limit the translocation of bacteria and their metabolites. In nonalcoholic steatohepatitis (NASH), on the one hand, the intestinal barriers are disrupted (thin mucus layer, decreased expression of tight junction proteins, altered ratio of Firmicutes to Bacteroidetes, dysbiosis, decreased short-chain fatty acids that result in increased leakage of bacteria and their metabolites (Lipopolysaccharide, MDP, flagellin, bacterial DNA) into the portal vein and systemic circulation, consequently stimulating the production of inflammatory cytokines in the systemic circulation. On the other hand, liver function is compromised because of the accumulation of fat, altered lipid metabolism, and increased microbial burden, which in turn elicits hepatic inflammation, hepatic stellate cell activation and collagen deposition, Kupffer cell activation, and triggering of the toll-like receptor 4 signaling pathway, which altogether contribute to the development of NASH. Tj: Tight junction; SCFAs: Short-chain fatty acids; KC: Kupffer cell; HSC: Hepatic stellate cell; LPS: Lipopolysaccharide; mLN: Mesenteric lymph nodes; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; TGF-β: Transforming growth factor-β; IL-1β: Interleukin-1β.

anaerobes *Bifidobacterium, Bacteroides* and *Clostridium,* which colonize most of the lower gut[4,5]. The neonatal microbiota is also influenced by the mode of feeding, where breast-fed babies show a more stable microbiota that has a higher copy number of *Bacteroides* and *Bifidobacterium* but a lower abundance of *Enterococcus* and *Streptococcus* species, while formula-fed babies have a higher abundance of *Clostridium, Streptococcus* and *Enterococcus*[6]. The early life microbiota only begins to take a semblance of adult microbiota when solid food is introduced and will remain relatively unstable until 3-5 years after birth[7]. The rapid expansion of early life microbiota and the adaptation to oxygen levels signify the earliest mechanisms for initiating gut microbial homeostasis[8].

The colon has the highest density of microbes in the gastrointestinal tract, harboring approximately 70% of all gut microbes, which are mostly members of the Firmicutes and Bacteroidetes phyla[9]. The Firmicutes to Bacteroidetes axis is important in maintaining gut homeostasis, as members of each phylum have specialized metabolic roles (*i.e.*, metabolism of sugar vs. indigestible fibers) that impact the microbiome and the host. It is believed that the role in homeostasis is optimized when the relative abundance is 80% Firmicutes and 15% Bacteroidetes[8,10,11]. However, the significance of this value and the actual impact it has on the host have been questioned by some researchers[12], emphasizing the importance of more research on the role of Firmicutes and Bacteroidetes in gut microbial homeostasis, health and disease. Nutrients, metabolic byproducts and the competition between exogenous microbes and commensals help prevent colonization of pathogens and maintain homeostasis. Different animal studies have shown that nutrient competition occurs between metabolically related microbiota members. For example, germ-free mice colonized with three human commensal strains of *Escherichia coli* (*E. coli* HS, *E. coli* Nissle 1917, *E. coli* MG1655) successfully prevented colonization of the cecum by the pathogen enterohaemorrhagic *Escherichia coli* (EHEC) EDL933, an *E. coli* 0157:H7 biotype, due to the three precolonized commensal biotypes outcompeting *E. coli* EDL933 for nutrients[13]. This colonization resistance was further shown to occur using multiple sugars as metabolic substrates for probiotic *E. coli* Nissle 1917 and commensal subtype *E. coli* HS, whose rapid growth effectively limited the colonization of EHEC *E. coli* EDL933 in a mouse model[14]. Competition for a shared nutritional niche of proline was

similarly demonstrated in a gnotobiotic mouse model colonized with early life microbiota where early-life *E. coli* 1 was shown to outcompete *E. coli* 0157:H7[15]. This colonization resistance was also thought to be attributed to the production of lactate and acetate by bifidobacteria and enterococci, which can suppress the motility of *E. coli* 0157:H7 under cecal anaerobic conditions[15]. Colonization resistance is also aided by the production of toxic antimicrobial peptides by commensals. For example, many members of the phylum Bacteroidetes produce toxic antimicrobial peptides through their type 6 secretion systems (T6SS)[16], *E. coli* produces narrow-spectrum antibiotics called microcins that effectively kill competitors within their niche[17,18], and the probiotic *Bifidobacterium* secretes broad-spectrum bacteriocins[19].

Overall, any extrinsic or intrinsic factors that upset the stable microbial communities will in essence destabilize the colonization resistance mechanisms and lead to disease by allowing colonization of pathogenic microbes and/or leakage of microbes and microbial toxins into circulation.

COLONIZATION RESISTANCE THROUGH MICROBIAL ENHANCEMENT OF GUT BARRIER FUNCTION

The gut is lined with a thick mucus layer made of a highly glycosylated mucin 2 protein, which is densely packed and insoluble in the layer closest to the epithelium but loosely packed and soluble on the outer layer[20,21]. This mucus layer prevents direct contact of bacteria with the gut epithelium, thereby reducing the potential for pathogen colonization[20,21]. The development of the mucus layer is enhanced by the gut microbiota and depends on the intestinal microbial composition. It has been shown that germ-free rodents have a much thinner mucus layer than their conventionally colonized counterparts[22]. Petersson and colleagues have shown that a thin colon mucosal layer in a colitis germ-free mouse model can be restored by administering lipopolysaccharides or peptidoglycans to germ-free mice[22]. Bacteria enhance the mucus layer in numerous ways, such as through the production of secondary metabolites. Short chain fatty acids (SCFAs), such as acetate produced by *Bifidobacterium* or butyrate produced by gram-positive Firmicutes such as *Faecalibacterium prausnitzii, Roseburia sp, and Butyricicoccus pullicaecorum*[23,24], are known to strengthen gut barrier function, normalize permeability, improve intestinal epithelium defense, protect against pathogenic infections, and reduce inflammation[25-28].

Intestinal epithelial cells are held together by a set of tight junction proteins that are molecules situated at the tight junctions of epithelial cells. The integrity of these tight junctions can be influenced by commensal bacteria and their effects on tight junction proteins. For example, *Lactobacillus rhamnosus* GG induces claudin-3 expression, *L. acidophilus* and *L. plantarum* stimulate the expression of occludin, and *Bifidobacterium infantis* preserves claudin-4 and occludin deposition at tight junctions[29,30]. In a mouse necrotizing enterocolitis model, *Bifidobacterium* was found to preserve claudin 4 and occludin localization in tight junctions, thereby preventing gut permeability[31]. In mouse models, probiotics have been shown to improve the integrity of the intestinal barrier, which has also been observed in Crohn's and colitis patients[28]. *In vitro* treatment of Caco-2 cells with the probiotic *E. coli* Nissle 1917 increases the expression and peripheral migration of ZO-2[32]. Treatment of Caco2 cells with the probiotic *Lactobacillus plantarum* MB452 increased occludin and cingulin gene expression[33]. These results indicate that *in vitro*, certain probiotics can improve gut barrier function. Maintaining the integrity of the intestinal barrier is essential due to the high levels of microbial lipopolysaccharide (LPS) present within the lumen of the gut, as LPS is a potent immunological signal that can induce an inflammatory cascade if detected systemically, which a healthy intestinal barrier effectively prevents[34]. Leakage of LPS and other microbial polypeptides into circulation due to dysbiosis can lead to inflammation in the liver (among other organs), which can lead to the development of liver disease[34].

COLONIZATION RESISTANCE AND BILE ACID METABOLISM

Pericentral hepatocytes primarily produce bile acids from cholesterol[35]. In humans, these acids are then transported to the gut, where they are dehydroxylated, epimerized, or dehydrogenated into different secondary bile acids, such as deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), ursocholic acid, or lithocholic acid (LCA)[35]. In mice, murideoxycholic acid and hyodeoxycholic acid are also produced[35]. Secondary bile acids are known to bind to the intestinal farnesoid X receptor (FXR) and G-protein coupled receptor 5 (TGR5)[36]. Some bile acid metabolites have also been shown to have a contradictory effect on gut barrier tight junctions[36]. UDCA and LCA, for example, have opposing effects on the barrier of human colonic T84 cells[36]. Treatment of these cells with primary bile acid-chenodeoxycholic acid (CDCA) combined with LCA leads to an increase in barrier permeability and the inflammatory cytokine IL-8[37]. Using a Caco-2 cell model, it was demonstrated that DCA led to an increase in the phosphorylation of epithelial growth factor receptor, which induced barrier dysfunction[38]. Prematurely weaned piglets treated with CDCA showed an improvement in the gut

barrier with higher ZO-1 expression and increased expression of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 and the anti-inflammatory cytokine IL-10[39]. The authors speculated that the anti-inflammatory effects of both IL-10 and ZO-1 counteracted the inflammatory effects of IL-6 and TNF-α, thus precipitating a net improvement in the intestinal barrier[39]. These examples demonstrate that bile acid metabolism is a significant key player in gut health, and it can be utilized as a therapeutic target for liver disease and other metabolic disorders, as will be discussed later.

MICROBIAL ASSOCIATION WITH LIVER DISEASE

Liver disease has been shown through preclinical and clinical trials to be accompanied by gut dysbiosis [40-44]. It has been shown that liver cirrhosis is also correlated with bacteremia, increased gut permeability, and increased circulatory LPS[43]. Dysbiosis has been noted in many mouse models of liver disease, such as secondary biliary fibrosis (common) induced by bile duct ligation, alcoholic liver disease induced by alcohol uptake in drinking water and hepatotoxicity-induced liver cirrhosis using carbon tetrachloride (CCL_4) treatment[42,43]. In humans, several gram-positive bacteria, including members of the genera *Clostridium* XI, *Anaerobacter*, *Streptococcus*, and *Lactobacillus,* were found to be more abundant in the gut in NAFLD patient biopsies than in healthy volunteers[45]. In contrast, *Oscillibacter* and *Flavonifractor* of the family *Ruminococcaceae* were abundant in healthy volunteers relative to NAFLD patients[45]. In severe fibrosis forms of NAFLD, the bacteria *Bacteroidetes vulgatus* and *Escherichia coli* were identified as the most abundant[46]. Although there has not yet been a general consensus on what microbial ratios of different strains exist in NAFLD patients, many research findings indicate that a lower Firmicutes to Bacteroidetes ratio is associated with liver disease[11,47]. Dysbiosis may be caused by a reduction in bile acids (which are bacteriostatic) of a cirrhotic liver, which precipitates inflammation and immunosuppression, factors that can positively feedback on cirrhosis[42]. Dysbiosis may also arise from increased saprophytic fungal growth in the alimentary canal. Cirrhotic liver patients who routinely receive antimicrobial treatment have an overgrowth of fungi, especially *Candida,* leading to fungal-bacterial balance in the gut and worsening dysbiosis[42]. Although cirrhosis is a systemic disease, it is believed to be worsened by dysbiosis both in the gut liver axis and outside this axis, such as in saliva and serum[42,48].

While there is a knowledge gap on the use of microbial interventions for NAFLD therapy, there are data showing that nonalcoholic steatohepatitis (NASH) patients improve following treatment with the antibiotic rifaximin, which is used for the treatment of traveler's diarrhea caused by *Escherichia coli*[49]. In a study examining the gut microbiota of stage 4 hepatitis C virus (HCV) patients, *Prevotella* and *Faecalibacterium* were found to be more abundant in HCV patients than in healthy controls, while *Ruminococcus* and some *Clostridium* species were more abundant in healthy controls than in HCV patients. *Bifidobacterium* was found only in healthy individuals[50]. Germ-free mice were shown to develop NAFLD following fecal microbial transplantation from donor hyperglycemic mice with systemic inflammation when fed a high-fat diet[51]. On the other hand, germ-free recipients that received fecal transplantation from normal donors (*i.e.*, normoglycemic with negligible systemic inflammation) did not develop NAFLD and were normoglycemic when fed a high-fat diet[51]. Rabot *et al*[52] also showed that germ-free mice fed a high-fat diet were more resistant to hepatic steatosis than colonized controls. In an experimental mouse model of cholestasis-induced liver fibrosis induced either through bile duct ligation or by CCl_4 treatments, colonization with complex microbiota (specific pathogen-free mice) was protective against severe fibrosis when compared to limited colonization (Altered Schaedler Flora)[53]. How the gut microbiota induces a leaky gut, bacteriaemia and an inflammatory flare leading to liver disease has been the subject of intense research. Brown and colleagues fed mice a high carbohydrate diet to induce a leaky gut[54]. This high carbohydrate diet caused a sloughing of the intestinal villi and reduced tight junction integrity, which allowed bacteria to translocate into the circulatory system[54]. In cirrhotic patients, it has been shown that microbial components leaking through the intestinal barrier, such as LPS, lipoteichoic acid, lipopolypeptides, and peptidoglycans, activate Toll-like receptors (TLRs) in hepatic stellate cells, Kupffer cells, and hepatocytes (all of which are differentially populated with TLRs 1-9), inducing severe inflammatory responses and fibrosis in the liver[43,55] Microbial activation of TLR2 in monocytes has especially been identified as significant in liver fibrosis through the production of TNF alpha, which initiates a cascade of reactions leading to increased gut permeability[43].

MICROBIAL METABOLITES IN LIVER DISEASE

Gut microbiota-host crosstalk in liver disease remains widely unclear. However, in recent years, many studies have established a correlation between different microbial metabolites and liver disease[56]. LPS are gut microbiota-derived endotoxins that form the major component of the gram-negative bacterial outer cell wall. High plasma levels of LPS have been identified in NAFLD patients and are associated with gram-negative intestinal bacterial overgrowth and compromised gut lining epithelial tight

junctions[57,58]. LPS induces an inflammatory response by activating hepatic Kupffer cells through TLR4. Apart from inducing proinflammatory cytokines and chemokines from hepatic Kupffer cells, LPS also activates hepatic stellate cells (HSCs) to differentiate into myofibroblast-like cells by producing extracellular matrix proteins, thus promoting liver fibrosis[49,59-61]. Other important metabolites are SCFAs from the fermentation of indigestible dietary fiber, which are mostly found in the colon, where most of them are produced and absorbed[62]. The major microbial fermentation products following microbial degradation of fiber are the SCFAs butyrate, propionate, and acetate. The body utilizes approximately 10% of the energy supply from microbially derived SCFAs, meaning that 90% is stored in white adipose tissue[63]. Several studies have revealed that gut microbial dysbiosis is associated with chronic liver diseases such as NAFLD or ALD[45,64]. In a metabolomic study in children with NASH, serum levels of 2-butanone and 4-methyl-2-pentanone were found to be elevated compared to those in healthy individuals[65]. Adults with NAFLD were found to have higher levels of fecal propionate and isobutyric acid, which are part of the fecal SCFA family[66]. Obese patients with NAFLD were also found to have high levels of propanoic acid and butanoic acid[67]. SCFAs such as acetate and butyrate modulate the host immune response by dampening the LPS-induced hepatocellular inflammatory response and restoring mucosal and systemic immunologic homeostasis, thus minimizing liver injury [68,69]. SCFAs can act as hormonal molecules by binding to G-protein-coupled receptors (GPCRs), which leads to activation of the GPCR pathway, slowing gut motility and increasing energy harvest[70-72]. Upon activation, glucagon-like peptide-1 is secreted from epithelial L-cells, enters circulation, and induces insulin release from the pancreas[70]. GPCR pathway activation also limits insulin-mediated hepatic and muscular fat accumulation and stimulates energy expenditure[71]. In adipocytes, SCFAs activate G protein-coupled receptor (GPR) 41 and GPR43 to inhibit lipolysis and activate adipocyte differentiation[70]. SCFAs also regulate immune cell functions through GPR43, which is widely expressed in most immune cells[73-75]. SCFAs have also been shown to inhibit histone deacetylases, which downregulate gene expression and reduce the production of inflammatory cytokines, particularly in macrophages and blood mononuclear cells during acute inflammatory hepatitis[69]. Therefore, it can be argued that dysbiosis that reduces microbial SCFA generation will result in a dysregulated inflammatory response and thus contribute to the progression of liver disease"

Indole and its derivatives are microbial metabolites of tryptophan breakdown. Indole upregulates tight junction proteins in the gut and downregulates colonic epithelium inflammatory genes through the aryl hydrocarbon receptor[76]. Indole-3-propionate activates pregnane X receptor to downregulate proinflammatory cytokine production and has been associated with protection against injury through oxidative stress signaling[76,77]. Indole-3-acetate has been shown to modulate hepatocyte lipogenesis, thus playing a protective role against NAFLD[78]. Microbial metabolism of dietary choline and L-carnitine produces trimethylamine (TMA), which is oxidized to trimethylamine N-oxide (TMAO) during hepatic detoxification of the blood through catalysis of the liver enzyme hepatic flavin monooxygenases[79]. TMAO is excreted in urine, and recent findings in animal NAFLD models fed a high-fat diet have shown increased urine levels of TMAO[80]. In a Chinese cohort study, the severity of NAFLD was closely associated with circulatory TMAO[81]. Bacteria are essential for the conversion of dietary choline to TMA, which is oxidized in the liver through the catalysis of hepatic flavin monooxygenase to generate trimethylamine-N-oxide, whose accumulation has been associated with both cardiac and renal disease[82,83]. Phosphatidylcholine is also metabolized by gut microbes to generate TMA, whose oxidation in the liver yields TMAO and, as previously described, may lead to kidney and cardiac disease[84,85]. It is now thought that accumulation of TMAO in the liver causes NASH through the inhibition of FXR and alteration of bile acid homeostasis[86]. SCFAs are significant microbial metabolites in the etiology of liver disease. More studies are required to target SCFAs as diagnostic or therapeutic tools for predicting or treating liver disease.

DIET AND XENOBIOTICS IN LIVER DISEASE

Liver disease is highly influenced by exposure to different environmental factors, which has recently been referred to as the exposome. It is now known that liver disease is impacted by an interaction between the genetic makeup of the host, exposome, and gut microbiome[87,88]. Certain types of gut microbiota have been associated with endogenous alcohol generation, which may in turn be hepatotoxic, leading to NASH[89]. The gut microbiota is important for the metabolism of bile acids, and in the absence or deficiency of bacteria that can convert primary bile acids to secondary bile acids, there is an accumulation of circulatory bile acids, which in turn activate TGR5, leading to monocyte dysfunction, which may exacerbate the hepatic inflammatory response and lead to liver disease[90]. High circulatory bile acids reflect a dysfunctional FXR, the nuclear receptor responsible for bile acid homeostasis, whose function is to facilitate enterohepatic bile acid circulation[91]. Dysbiosis affecting 7α-dehydroxylation-rich Firmicutes, which convert primary bile acids to FXR-low-binding secondary bile acids, will inevitably affect the function of FXR, leading to liver disease[92]. The liver is a crucial filter for toxins that find their way into the body either accidentally or deliberately. Alcohol is by far the most significant xenobiotic causing liver disease in humans, and it has been identified as the cause of ALD

[93]. It can be argued that alcohol consumption causes both destruction of microbial communities and rupture of the barrier wall integrity in the gut and leads to induction of inflammation during detoxification in the liver. A compromised gut barrier leads to leakage of LPS and other microbial ligands into circulation, triggering inflammation of liver cells.

A high-fat diet and environmental pollutants are further risk factors for liver disease, and their effects are exacerbated by microbial metabolites[94,95]. It is likely that most xenobiotics, in addition to being directly toxic to hepatic cells, will cause dysbiosis that favors changes in microbial composition that generate toxic liver disease-causing metabolites. The changes in these microbial metabolites may therefore be used as noninvasive diagnostic biomarkers for liver disease[96] but may also become significant therapeutic targets for the treatment of this disease[96-98]. A high carbohydrate diet has been demonstrated in environmental enteropathy animal models to lead to intestinal wall epithelial brush-border shortening and loosening of tight junctions[54]. Furthermore, small intestinal gram-negative bacterial overgrowth and high plasma LPS levels can lead to liver disease[56]. In the absence of dietary fibers, the gut microbiota cannot produce sufficient SCFAs, which may lead to a dysregulated inflammatory response and liver disease[99,100]. Most liver metabolism occurs through the catalysis of cytochrome P-450 (CYP-450), and it is known that many dietary biproducts can influence the activity of CYP-450[101]. Dietary retinoids, for example, are metabolized by hepatic cells, including hepatic stellate cells. An alteration in the uptake and metabolism of retinoids may influence retinoic acid signaling, which may activate hepatic stellate cells, resulting in loss of retinoid stores, aberrant extracellular matrix generation and the onset of fibrosis, which inevitably precipitate liver disease[102]. Additionally, alcohol consumption affects hepatic retinoid metabolism through inhibition of retinoid oxidation, induction of CYP2E1 enzymes to increase retinoic acid metabolism, or increased peripheral tissue damping of retinoic acid, all of which leads to activation of hepatic stellate cells and development of liver disease[103]. Retinoic acid is a gut microbial metabolite of vitamin A whose intestinal concentration is modulated by suppression of retinol dehydrogenase 7 expression by commensal *Clostridia* microbes[104]. Retinoic acid not only regulates bile acid homeostasis but also shares with it the receptors retinoid X receptor and FXR and therefore shares the functions of lipid metabolism and insulin sensitivity[105]. In a rat model, a high-fat diet in combination with high glucocorticoid treatment resulted in a fourfold hepatic lipid deposition and an almost threefold increase in circulatory alanine aminotransferase indicative of liver injury[106]. A high-fat diet also caused severe liver damage with high levels of circulatory alanine transaminases (ALT) and aspartate aminotransferases (AST) in a mouse model[107]. Mice fed a high-fat diet developed high intestinal gram-negative microbial growth and an increase in ethanol-producing bacteria when compared to mice fed normal chow[107]. This result is consistent with findings from clinical studies where it has been documented that microbial diversity rapidly changes with a change in diet[108]. Therefore, it can be concluded that diet, food additives, and xenobiotics affect liver disease by influencing gut microbial composition, gut permeability, and microbial metabolites. The liver plays a major role in metabolism and blood detoxification and is thus prone to damage from microbial endotoxins, environmental toxins, and microbial dietary metabolites, all of which work together in cascaded inflammatory responses to cause liver injury. Understanding the individualized microbial signatures and their influence on gut permeability, immunologic inflammatory responses, and the hepatic response to insult will expose multientry avenues to precision liver disease therapy.

MICROBIOME-HOST INTERACTION IN LIVER DISEASE

The intestine is heavily colonized with microbiota, yet the surrounding tissues remain sterile. This barrier is maintained by intricate crosstalk between gut microbes, the gut wall epithelium, and the innate immune system[109,110]. The expression of intercellular tight junction proteins between the intestinal epithelium is regulated by cytokines such as interferon gamma and TNF and other regulatory cytokines that interact with immunoglobulin A (IgA)-coated gut microbiota to maintain gut and immune homeostasis[111]. A change in diet or intake of xenobiotics such as alcohol, prescription/over-the-counter drugs, or other environmental chemicals may lead to destabilization of the intestinal homeostatic environment either through selective overgrowth or reduction of specific microbial strains or injury to the mucosal lining. A destabilization of the homeostatic environment will give way to a shift in the immunological signaling molecules protective to the gut tight junctions and a sloughing of intestinal villi. This breach in the barrier allows leakage of microbial endotoxins into circulation and microbial translocation into the liver, thus triggering an immunological inflammatory response once the microbial products are detected by the liver's pathogen recognition receptors, mainly the TLR and nucleotide oligomerization domain-like receptors[109,112]. HSCs are endowed with TLR 2, 4, and 9, which are associated with promoting TLR4 fibrosis[43,60]. Kupffer cells are lined with TLR 2, 3, 4, and 9 and are hepatic macrophages that form the main targets of microbial ligands within the liver[43]. Furthermore, hepatocytes express TLR 1-9 and are the most abundant cells in the liver, playing a critical role in the acute phase of the immunologic response through cytokine-like IL-6[113]. The inflammatory response of the liver to leaked gut-microbial endotoxins is not yet fully understood. However, it is

known that upon activation, Kupffer cells release proinflammatory and profibrogenic cytokines, such as TNF-α, Transforming Growth Factor (TGF)-β and IL-1β, and a few more members of the inflammasome whose effect is to induce inflammation and accumulation of lipids in the liver, and if this is not resolved, it leads to fibrosis NAFLD[114]. Therapeutic target efforts are geared toward minimizing the hepatic inflammation seen after proinflammatory cytokine release. Chemokine receptor antagonists such as C-C motif chemochine receptor (CCR) 2 and CCR5 [Cenicrivinoc (CVC)] have been used with some success to decrease leukocyte infiltration, and when used in a diet-induced NASH mouse model and a thioacetamide-induced fibrosis rat model, liver fibrosis was effectively reduced[115,116]. This outcome has since been replicated in phase 2 clinical trials with a remarkable reduction in fibrosis[117]. Several other proinflammatory cytokines, including IL-17, IL-11, and IL-1, are still under investigation. A clinical trial therapy utilizing an IL-1 pathway anti-inflammatory drug, diacerelin, achieved a remarkable reduction in fibrosis in NAFLD patients with diabetes[118].

THE GUT MICROBIOME AS A DIAGNOSTIC BIOMARKER FOR LIVER DISEASE

The dynamics of the gut microbiome could be used as a noninvasive diagnostic tool for liver cirrhosis and hepatocellular carcinoma (HCC)[119]. In a cross-regional prospective validation study in China, human fecal samples analyzed for microbial diversity revealed a significant rise in diversity as the liver condition advanced from cirrhosis to HCC with cirrhosis[119]. There was also a high level of butyrate-producing bacteria in healthy controls relative to early cirrhosis patients and a notable rise in LPS-producing bacteria in HCC patients[119]. In a different experiment, gut microbiota known to originate from the oral cavity were found to be enriched in liver cirrhosis patients relative to healthy volunteers [120]. In an Asian NAFLD cohort, *Ruminococcaceae* and *Veillonellaceae* species were found to be more predominant in NAFLD patients relative to healthy individuals[121]. These microbiome changes could not be associated with genetic predispositions known to influence NAFLD and were thought to be environmentally driven[121]. *Bacteroides* and *Escherichia* spp. have, on the other hand, been associated with liver fibrosis in NAFLD patients[122]. Overall, these multiregional studies indicate that there is great potential for the gut microbiota as a noninvasive diagnostic biomarker for liver disease with distinct indications of the staging of fibrosis and inflammation[121,123]. There is also great potential for the gut microbiota and associated metabolites to be utilized as therapeutic biomarkers[119-121]. It must, however, be appreciated that as of yet, a single microbial signature indicative of liver disease does not exist mainly because disease outcome is influenced by multiple factors such as diet, genetic background, age, and lifestyle (such as alcohol consumption), all of which must be considered while interpreting data on the predictive value of fecal microbiota on liver disease[124].

THERAPEUTIC APPROACHES

As we have discussed above, dysbiosis and a dysfunctional gut barrier promote the leakage of microbial endotoxins and components, as well as bile acid metabolites, into circulation, which can eventually lead to liver injury. Various therapeutic approaches (which are at various stages of testing) could be used to address these different factors for the treatment or prevention of liver disease, which will be highlighted below. Although SCFA supplements could be an attractive therapeutic approach in liver disease, their taste is normally not well tolerated. However, methods such as microencapsulation[125], either as soft gels or liquid capsules, are available that mask the taste of bitter medications and could be used for oral delivery of SCFA, which has the added benefit of being slow release and helps prevent evaporation of some volatile SCFAs, such as butyrate. Butyrate enemas have been used in a rat model, with the treatment group showing improved mucosal repair and reduced colonic damage compared to the untreated control groups[126]. However, butyrate enemas did not show any improvement in clinical studies with ulcerative colitis patients[127]. There is potential for the use of SCFA as a therapeutic approach, but more research is required to develop an optimal approach. Prebiotics such as inulin represent a substitute approach for the supply of SCFAs[98]. Multiple agonists of FXR are under investigation, including GS-9674 and LJN452, in phase 2 trials for NASH[98]. Some fibroblast growth factors (FGFs), such as FGF19 and FGF21, have shown encouraging results for NAFLD therapy[128,129].

Probiotic interventions

Treating dysbiosis and restoring homeostasis is complicated due to the wide range of associated factors that lead to a loss of important microbial populations or diversity in the first place. In most cases, treating dysbiosis with a single approach usually gives discouraging outcomes. However, studies involving probiotics have shown encouraging results in terms of safety, tolerance, and efficacy[130]. In a Phase 1 clinical trial, *Lactobacillus rhamnosus* GG administered to cirrhotic patients resulted in reduced *Enterobacteraceae* and increased relative abundance of *Clostridiales incertae* Sedis XIV and *Lachnospiriceae* with reduced endotoxemia and decreased pathogenic bacterial growth indicative of improved health

[131]. In another study using multiple probiotic strains, a reduction in inflammatory cytokine flares in cirrhotic patients was observed[132]. In obese, sonographically identified NAFLD children, treatment with a probiotic combination of *Bifidobacteria (B. bifidum and B. lactis)* and two *Lactobacilli* (*L. rhamnosus* DSMZ 21690 and *L. acidoph*ilus) strains significantly lowered intrahepatic fat content and ALT levels as well as AST relative to the placebo treatment[133]. This reduction in hepatic steatosis was replicated in NAFLD patients treated with a multistrain probiotic[134]. In another study, a twelve-week treatment of 30 NAFLD volunteers with six strains of bacteria containing *Bifidobacterium breve* and *B. lactis*, *Lactobacillus rhamnosus, L. acidophilus* and *L. paracasei pacasei* and *Pediococcus pentosaceus* in a randomized, double-blind, placebo-controlled study led to an improvement in proinflammatory cytokines, a reduction in cholesterol and a decrease in body weight[135]. When probiotics are mixed with compatible prebiotics, better outcomes have been achieved in clinical trials, but more studies are needed to determine the most effective combinations[136,137]. Hepatic steatosis has, for example, been reported to decrease in patients with NASH following symbiotic and prebiotic treatment. Serum alkaline phosphatase was decreased following treatment with probiotics, prebiotics and synbiotics[136] However, it is noteworthy that the outcomes are dependent on the composition of probiotics, the exposure time, and the dosage[136]. Studies in animal models have shown similar outcomes as in human studies. In rats fed a high-fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool[138]. In addition, hepatic steatosis was markedly reduced after 12 wk of treatment with *B. longum*, but this was not the case with *L. acidophilus* treatment[138]. In a diabetic rat model, treatment with *Akkermansia muciniphila* led to a decreased inflammatory response and improved liver function[139]. In hepatic encephalopathy, a mixture of *Lactobacillus plantarum*, *L. casei, L. delbrueckii* subsp. Bulgaricus, *Bifidobacterium infantis, B. longum, B, breve, and Streptococcus salivarius* subsp. Thermophilius has been associated with both primary and secondary prophylaxis[140,141]. Yogurts containing *L. bulgaricus, S. thermophilus, L. acidopilus* La5 and *B. lactis* Bb12 as well as a prebiotic mixture of fruco-oligosaccharides and *L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum,* and *L. bulgaricus* have been shown to improve aminotransferase in NAFLD patients[142-144]. In NASH patients, probiotics containing *L. bulgaricus* and *S. thermophilus* have also shown improvement in aminotransferase[145]. A combination of *B. longum* W11 and fructooligosaccharides, on the other hand, has shown improvement in aminotransferase and the histological score activity of NASH patients[146]

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the administration of a solution containing fecal material from a "healthy" donor into the intestinal tract of a recipient to modify that recipient's gut microbial composition for targeted health benefits[147]. To date, FMT has been successfully used in the treatment of recurrent *Clostridium difficile* infection, and there is growing evidence that FMT can be used to treat noninfectious diseases such as inflammatory bowel disease, obesity, and other metabolic disorders [147]. FMT has also been tried as a therapeutic option for liver disease. In a diet-induced steatohepatitis mouse model, FMT-treated mice showed increased SCFAs, improved expression of tight junction proteins, reduced proinflammatory cytokines and less intrahepatic lipid deposition compared to controls (*i.e.*, no FMT)[148]. There have also been several human clinical trials but with mixed outcomes, with some achieving a significant reduction in proinflammatory cytokines and improved gut barrier function and others not responding to therapy[149,150]. Future experiments should address the question of who qualifies as a healthy donor, how should we deal with the variation in gut microbial diversity among the recipients, and how best to package the product for better acceptability.

Bile acid metabolism

A recent study in mice indicated that during antibiotic-induced dysbiosis, the homeostasis of bile acids was equally destabilized[151,152]. Treatment of these mice with flavanones and total phenolic extracts of citrus aurantium L. (TPE-CA) restored bile acid homeostasis and gut barrier integrity[152]. TPE-CA also regulates the enterohepatic circulation entry of bile acids through the farnesoid X receptor-fibroblast-growth factor 15 pathway[152]. The effects of dysbiosis and increased intestinal unconjugated bile acid that are observed in ALD were reversed through improved FXR activity and gut barrier function following treatment with fexaramine, which is an intestine restricted FXR agonist. These results indicate that modulation of cyp7a1 and lipid metabolism can be achieved in a mouse model and thereby minimize ethanol-derived liver damage by targeting the bile acid-FXR-fibroblast-growth factor 15 signaling pathway[153]. Future experiments to verify these findings in higher mammals and translate the results to therapeutic interventions for human liver disease are warranted.

Precision microbial engineering

The mechanisms by which the intestinal microbiota influences the development and/or progression of liver disease are only beginning to unfold, but to fully elucidate the microbiome role in liver disease, a more comprehensive picture of the dynamics of the gut ecosystem is needed. Unfortunately, most of our knowledge about the intestinal microbiota arises from fecal or biopsy sample analysis, which is not

representative of the entire gut microbiome. However, novel technologies are being developed to address this knowledge gap. One such innovation is a capsule sampler and drug delivery system that is swallowed and utilizes mechanical gut peristaltic movements to guide the capsule down the entire length of the gut as the capsule collects samples[154]. Recently, a capsule robot was designed from a shape memory alloy spring with a chamber of a storage capacity of 500 μL, which showed enhanced sample preservation[155]. Another approach consists of an inexpensive 3D-printed sampler containing a hydrogel whose swelling ability seal and protects the liquid gut samples[156]. Such strategies that analyze small samples from various sites will provide information on microbiota distribution and will make microbial engineering and microbial targeting more feasible.

One such microbial engineering approach being developed is the use of Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) Cas-based instructions to precisely cut off targeted genetic sequences of the microbial genome and thus change their function *in vivo*[157]. A conjugative plasmid, TP114, was recently used as a delivery vehicle for CRISPR-Cas9, targeted at drug-resistant *Escherichia coli* and *Citrobacter rodentium*, which led to full clearance of these organisms in a mouse model four days after administration[157]. More recent delivery systems for CRISPR-Cas9 have been designed to utilize probiotics as a genetically engineered conjugative vehicle that are more efficient and practical to use than bacteriophage-based systems[158,157]. The use of CRISPR-Cas9 as antimicrobial therapy is still in its early stages but has the potential to be an effective therapy for targeting specific, undesired microbes in the dysbiotic gut of liver disease. Other approaches to manipulate the gut microbiome are mucosal vaccines. IgA is the predominant antibody in the gut that binds to pathogens and commensals, preventing their translocation across the mucosal barrier. Using a probiotic-based mucosal vaccine with *Lactobacillus acidophilus*, Fox *et al*[159] showed that a potent, diverse IgA response could be elicited which could help with colonization resistance. In another study, Slack and colleagues designed an oral vaccine using genetically modified *Salmonella enterica* capable of setting evolutionary traps for prophylaxis treatment in a mouse model[160,161]. While this technology was advanced into a pig model and is currently being tested on human neonates to treat neonatal sepsis and necrotizing enterocolitis, it has hallmarks to be equally beneficial as therapeutic approaches for liver disease.

Diet and lifestyle changes as therapeutic targets

There are many therapeutic options for NAFLD that are being explored, some of which are in advanced levels of clinical trials; however, no treatment is yet available[124]. Diet and lifestyle changes remain the most effective methods of managing liver disease[162]. Low caloric diets, low carbohydrate intake and low protein diets have all been shown to be effective in the management of liver disease[163,162]. It should, however, be noted that dietary changes alone cannot achieve the intended long-term weight loss goals to reduce liver inflammation. It is rather a combination of correct diet and exercise that is most effective against NAFLD[162]. The response to dietary changes and exercise on both gut microbiota that are negatively associated with liver disease and the amount of fat in the liver is different between individuals and between races[164] The amount of *Bacteroides*, for example, is lower in Chinese NAFLD individuals after diet and exercise compared to people from the West, and this is correlated with lower hepatic fat[164]. It has also been noted that Bacteroides increases in obese volunteers but decreases in lean volunteers following exercise and diet intervention[165]. This is suggestive of personalized intervention approaches of diet and lifestyle changes[164].

CONCLUSION

The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms by which the microbiome contributes to liver disease are unknown, there is strong evidence that translocation of various metabolites across the mucosal barrier plays a strong role, which is precipitated by a dysbiotic gut microbiota. Considering the importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. More research needs to be done to understand the crosstalk between the microbiome and host as it relates to liver disease so that more effective and targeted preventative and therapeutic options can be developed.

FOOTNOTES

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REVIEW

Radiological findings in non-surgical recurrent hepatocellular carcinoma: From locoregional treatments to immunotherapy

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Abstract

Since hepatocellular carcinoma (HCC) represents an important cause of mortality and morbidity all over the world. Currently, it is fundamental not only to achieve a curative treatment but also to manage in the best way any possible recurrence. Even if the latest update of the Barcelona Clinic Liver Cancer guidelines for HCC treatment has introduced new locoregional techniques and confirmed others as well-established clinical practices, there is still no consensus about the treatment of recurrent HCC (RHCC). Locoregional treatments and medical therapy represent two of the most widely accepted approaches for disease control, especially in the advanced stage of liver disease. Different medical treatments are now approved, and others are under investigation. On this basis, radiology plays a central role in the diagnosis of RHCC and the assessment of response to locoregional treatments and medical therapy for RHCC. This review summarized the actual clinical practice by underlining the importance of the radiological approach both in the diagnosis and treatment of RHCC.

Key Words: Carcinoma; Hepatocellular; Liver; Ablation; Catheter; Radio frequency ablation; Ablation techniques; Medication therapy management; RECIST

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Core Tip: During the follow-up of patients affected by hepatocellular carcinoma (HCC), radiology is considered the key to the diagnosis of recurrence, by taking advantage of cross-sectional imaging with a special focus on computed tomography and magnetic resonance imaging. As in the case of active surveillance in a patient with mild to moderate risk for developing HCC, cross-section imaging can help in the quick identification of signs of recurrence. Moreover, radiology plays a key role in the evaluation of treatment response during medical therapy for HCC, recently approved in the revised version of the Barcelona Clinic Liver Cancer staging.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth-leading cause of cancer-related deaths worldwide, and it is the most frequent primary liver tumor, accounting for about 85% of primary liver malignancies. Cirrhosis is the histological substrate on which 80% of HCCs arise[1]. According to the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases, all patients with a high risk of developing HCC should undergo a surveillance program[2,3]. Treatment options with curative intent are liver resection (LR), locoregional treatments (LRT), or orthotopic liver transplantation (OLT), and the choice of treatment is influenced by intrinsic features of the lesion, aspects related to the patient, and medical and economic resources available in each center[4,5].

Many HCCs are detected at an intermediate or advanced stage, which are not eligible, at least in the first instance, for curative treatment. In such cases, several treatment options are available, which can also be used in a combined or sequential manner including local termoablation [radiofrequency ablation (RFA), microwave ablation (MWA)], traditional transarterial embolization with traditional chemotherapy or microparticles [transcatheter arterial embolization, transarterial chemoembolization (TACE), TACE with drug-eluting beads (DEB-TACE)], transarterial radioembolization (TARE), and stereotactic ablative radiotherapy[6]. Finally, in cases of metastatic disease, the most common and widely used and approved approach remains systemic therapy with sorafenib, a tyrosine kinase inhibitor drug implicated in several pathogenetic mechanisms[7].

However, even if the primary goal is to have a curative intent, recurrence rate after transplantation is between 8% and 21% despite the use of new predictive models[8]. By contrast to OLT, both LRT and LR suffer from a high recurrence rate (60%-80%). When occurring, tumor recurrence may be considered non-transplantable if it exceeds the transplantation criteria such as those defined by the alpha-fetoprotein or Milan/up-to-seven criteria. Non-transplantable recurrence is a major cause of precluding salvage OLT, which showed comparable overall survival (OS) to primary OLT in patients with HCC with compensated cirrhosis[9].

Even if the latest update of the Barcelona Clinic Liver Cancer (BCLC) guidelines[10] for HCC treatment has introduced new locoregional techniques and confirmed others as well-established clinical practices, there is still no consensus about the treatment of recurrent HCC (RHCC)[11]. For these reasons, the multidisciplinary approach should be considered to define the best option for each RHCC patient[12]. On this basis, this review summarized the actual clinical practice by underlining the importance of the radiological approach both in the diagnosis and treatment of RHCC.

LRT

To date, the available options for RHCC were similar to naïve-HCC options and include LR, OLT, and LRT for patients with liver-only recurrence, TACE, TARE, and stereotactic ablative radiotherapy for patients with unresectable disease, and systemic therapies or enrollment in clinical trials for patients with extrahepatic disease recurrence[13-15].

Ablative treatments

Since only 15%-30% of patients with RHCC are suitable for an LR due to progressive liver dysfunction,

presence of multiple nodules, tumor location, or donor shortage for LT, the ablative treatments play a crucial role in early-stage RHCC[16]. RFA for RHCC is a safe and feasible technique, offering no significant difference in OS compared to RFA for primary HCC[17]. As both RFA and LR are indicated in RHCC tumors with similar features, many studies have compared the two treatments.

Three interesting and recent meta-analyses[13,18] established that LR provided better outcomes than RFA, especially in long-term survival outcomes. RFA is associated with a decreased risk of major complications and requires shorter hospitalization time, a more cost-effective approach in comparison with LR. Moreover, in well-selected patients, RFA may be an optimal choice for RHCC with similar outcomes of LR, notably for a single lesion < 3 cm or in patients with three or fewer nodules, following the guidelines for primary HCC[10]. Also, other studies, including one randomized controlled trial[19], confirmed the same results[20-22].

RFA performances are found to be worse than LR in disease free-survival (DFS), because the LR may ensure removal of the tumor-bearing portal territory where micrometastases and microscopic vascular invasion are present and usually impossible to detect through external ultrasonography[13].

To overcome the shortcomings of RFA, MWA has been assessed in the treatment of HCC, as it produces significantly larger areas of necrosis, faster ablation times, higher intratumor temperature, less tumor seeding risk, and less susceptibility to heat-sink effect over RFA[15,23] (Figure 1). However, there are few studies about percutaneous MWA performance in RHCC. Only one has compared surgical MWA and LR for RHCC showing the safety and feasibility of surgical MWA for RHCC within 3 cm in size and no more than three nodules[24]. Nevertheless, MWA was proven to be superior to RFA[25] and competing with LR when the tumor is > 3 cm and < 5 cm and close to the large vessels[26]. During treatment of very early and early HCC, RFA, MWA, and cryoablation have substantially similar outcomes[23].

A multicentric randomized controlled trial comparing RFA with cryoablation in HCC < 4 cm reported no differences in terms of OS and DFS but found differences regarding local tumor control in favor of cryoablation (7.7% *vs* 18.2%, $P = 0.04$)[27]. While another study conducted on 3239 patients showed a significant advantage in liver cancer-specific survival for RFA[28]. Therefore, the results regarding cryoablation are still unclear[29]. However, data are currently lacking concerning outcomes following the use of cryoablation in RHCC, and future studies should be focused on these aspects.

TACE

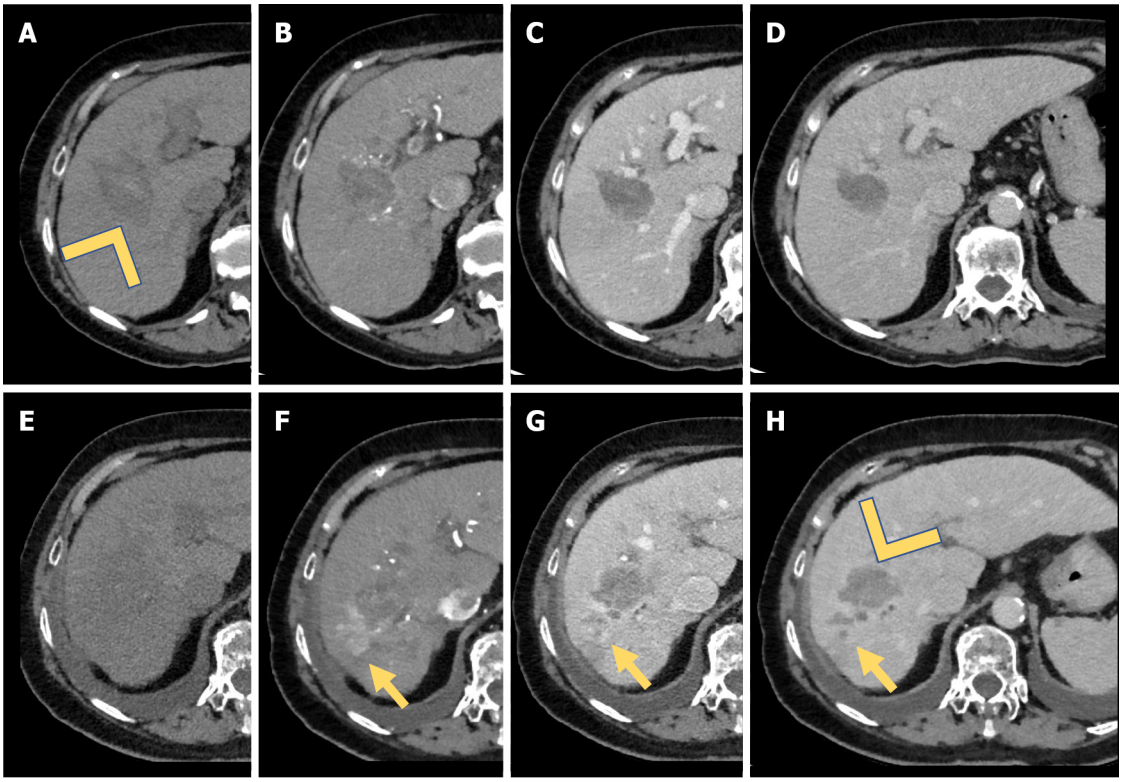
TACE is the most common treatment modality used for RHCC following initial resection[16,17]. However, as with LR, appropriate candidates for TACE should be carefully chosen based on their hepatic reserve[16,30] (Figure 2). However, there may exist a significant risk of worsened liver dysfunction following TACE among patients who have undergone prior hepatectomy[15,16,30]. Scores such as up-to-seven criteria or biomarkers such as Mac-2 binding protein glycosylation isomer to assess liver fibrosis can be used to identify patients who tolerate TACE less[16,30].

Regarding TACE in RHCC, Zu *et al*[31] demonstrated that the 1-, 2-, and 3-year OS rates after TACE were 73%, 52%, and 32%, respectively, while the number of resected HCC nodules (≥ 2), size (> 5 cm) of the RHCCs, and the number of TACE sessions (≤ 3) are independent risk factors for poor outcomes after TACE for recurrent HCC. Comparing TACE in naïve-HCC and RHCC, Liu *et al*[32] showed that RHCC treated with TACE accomplished acceptable results. After the propensity score matching analysis, there were no statistically significant differences between the naïve-HCC group and RHCC group in objective tumor regression and disease control rate. On the other side, the RHCC group had a shorter median OS (24 mo *vs* 33 mo) and PFS (10 mo *vs* 12 mo) in comparison with the naïve-HCC group.

Since it is a non-curative treatment, a recent meta-analysis demonstrated that TACE had worse outcomes (OS and DFS) than liver transplantation, LR, and RFA in RHCC patients[33]. Even comparing the two LRTs, Gou *et al*[34] showed that RFA had better short-term and long-term OS than TACE. Conversely, TACE may improve survival in patients with inoperable tumors, with large lesions or multifocal RHCC (beyond the Milan Criteria), and early (< 1 year) recurrence[35,36]. Interestingly, TACE proved to be a more effective option than LR/RFA in RHCC of BCLC stage 0 or A with microvascular invasion, especially in those that recur early after curative resection[37].

Among transarterial procedures, DEB-TACE, which uses doxorubicin, and TARE, using yttrium-90-labeled spheres, have been developed[12]. Even if it has been demonstrated that DEB-TACE facilitates higher concentrations of drugs within the target tumor and lower systemic concentrations with fewer adverse events than conventional-TACE in the management of HCC, especially on RHCC, there is no strong evidence showing the superiority of DEB-TACE over conventional TACE[38,39]. There are a lack of studies considering DEB-TACE as monotherapy for RHCC.

TARE may be an option for intermediate or advanced-stage HCC. It could also be used as an alternative to TACE especially for patients with portal vein thrombosis or for patients with earlier stages who are not eligible for curative procedures[16]. It is a safe and effective procedure for RHCC following LR, with satisfactory outcomes (median time-to-progression and OS were 11.3 mo and 22.1 mo, respectively)[40].



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Figure 1 Computed tomography study for assessment of treatment response (after microwave ablation). A: A 65-year-old male underwent microwave ablation of a hepatocellular carcinoma located in the V-VIII hepatic segment. Computed tomography scans were acquired after 2 wk of treatment. A large hypoattenuating area in the unenhanced (arrowhead) phase located in the V-VIII hepatic segment represented the treatment zone; B-D: During the dynamic study, no enhancement during the arterial phase (B) was seen, underlying the complete treatment response. Also, during the portal venous phase (C) and delayed phase (D) no wash-out was seen; E-H: After 1 year, the area of treatment was less hypoattenuating in the unenhanced phase (E), with a pseudonodular peripheral area of hypervascularization during the arterial phase (F, yellow arrow), with a wash-out during the portal venous and delayed phases (G and H, yellow arrow). On the other hand, the area of treatment did not show any arterial phase hyperenhancement or wash-out (H, arrowhead). The final diagnosis was hepatocellular carcinoma recurrence after microwave ablation (yellow arrows).

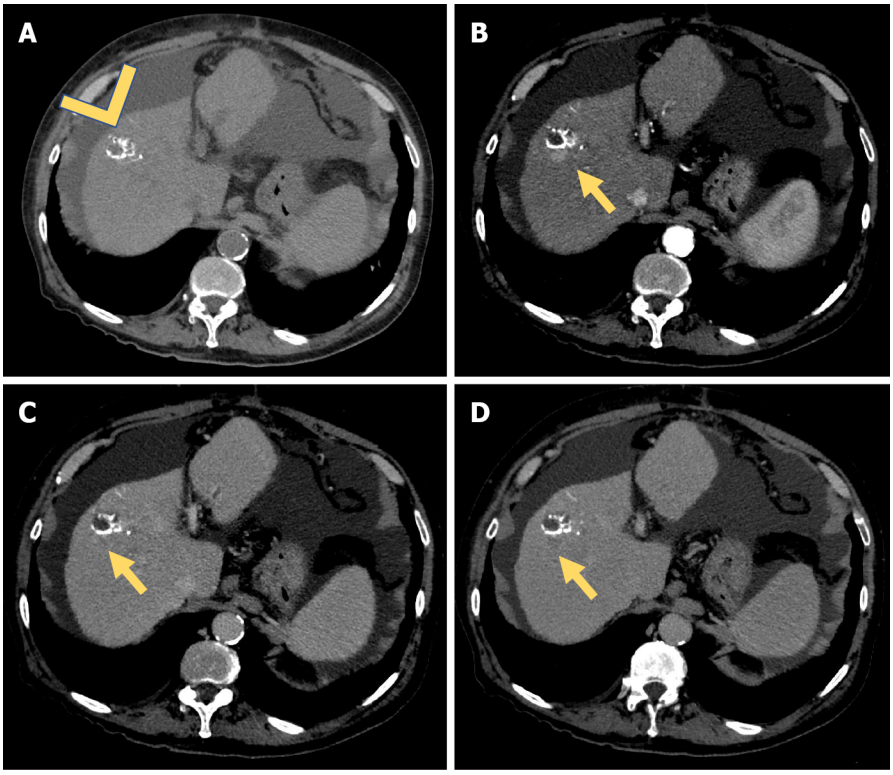
Combined therapies

Since RHCC frequently requires aggressive treatment to reach good therapeutic outcomes, the combined approaches have been evaluated by several studies for RHCC[16]. It has been proven that TACE alone is unable to cause complete tumor necrosis[41] and that RFA cannot detect satellite lesions [13]. Therefore, combined therapies may have a synergistic effect and be beneficial for patients with RHCC. TACE-RFA combined treatment can cause tumor necrosis up to 7 cm in diameter in one session [42].

The combination of TACE and RFA leads to theoretical advantages over either monotherapy. TACE can reduce the heat sink effect of the RFA, thereby increasing the ablation range. On the other hand, satellite lesions can be detected through TACE[41]. Furthermore, TACE with the intralesional accumulation of radio-opaque iodized oil used or drug-eluting beads increases the echogenicity and conspicuity of small HCC, otherwise hardly visible on ultrasound (US) guidance during RFA[43].

Song *et al*[44] showed that TACE-RFA had better DFS in comparison with TACE alone in patients with RHCC ≤ 5 cm. However, there were no significant differences between the two groups in OS and adverse events. Ascites is a frequent complication in the TACE-RFA group (Figure 3). Moreover, TACE-RFA provides comparable local efficacy and long-term survival results for patients with RHCC after hepatectomy, both for tumor size < 5 cm and > 5 cm. Furthermore, the TACE-RFA group has fewer complications[41,45] and lower hospitalization time in comparison with the LR group[45].

Zhang *et al*[46] demonstrated that DEB-TACE combined with RFA can increase the survival of patients with RHCC. Notably, OS rates were similar to primary HCC, while DFS rates were lower. A recent study[47] comparing MWA-TACE with TACE alone for small RHCC showed that the 5-year PFS of the combined therapy (37.5%) was higher than that of patients receiving TACE alone (18.7%), while the cumulative OS rates at 5 years were 61.1% for TACE-MWA and 50.3% for TACE alone, with no significant differences. Song *et al*[44] and Ji *et al*[47] demonstrated that combined therapies improve tumor control but not long-term survival outcomes.



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Figure 2 Computed tomography study for assessment of treatment response (after transarterial chemoembolization). A: A 55-year-old male underwent conventional transarterial chemoembolization of a hepatocellular carcinoma lesion located in the VIII hepatic segment. Two years after treatment, a computed tomography scan showed areas of hyperattenuating components in the unenhanced phase (A), representing the ethiodized oil (arrowhead); B-D: During the arterial (B) phase, a pseudonodular area of hypervascularization during the arterial phase (B, yellow arrow) was seen, with a slight hypoattenuating appearance during the portal venous phase (C) and a clear washout during the delayed phase (D). This represents an example of recurrent hepatocellular carcinoma after transarterial chemoembolization.

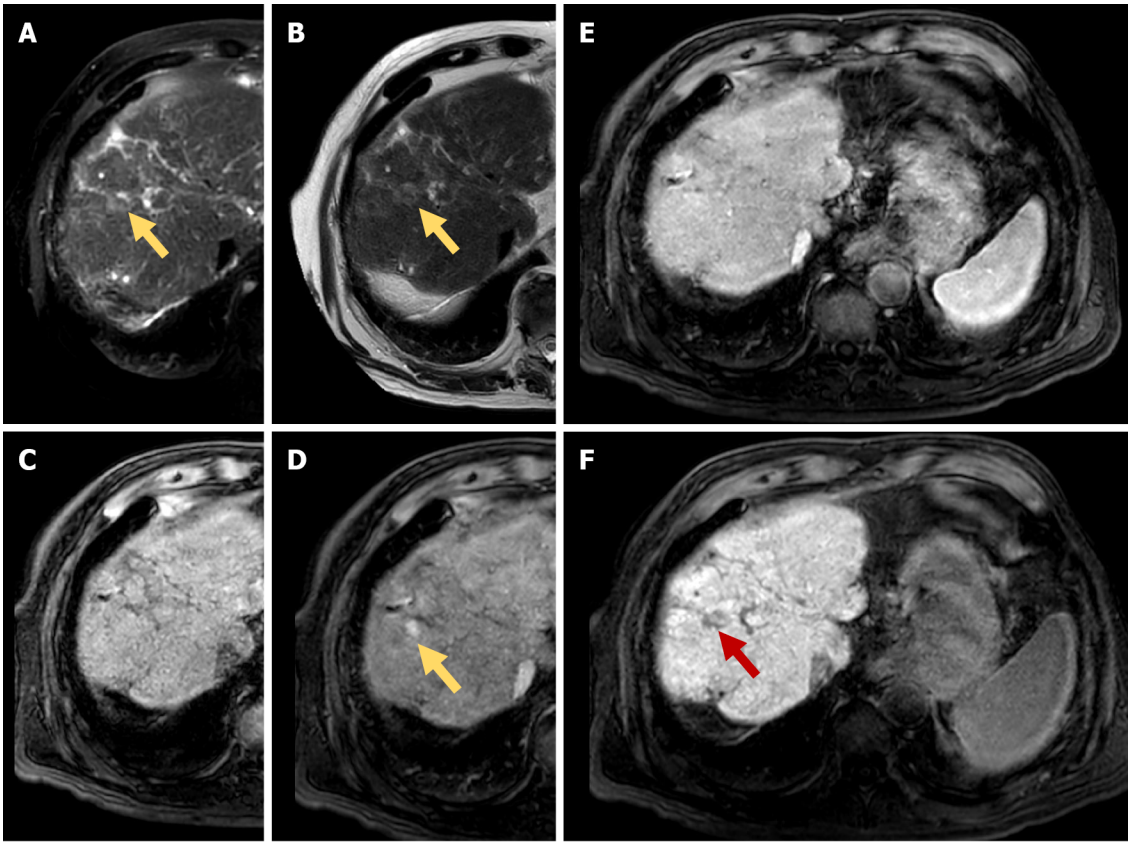
MEDICAL THERAPY

Since 2007, sorafenib represented the standard medical treatment of advanced HCC[48] (Figure 4). Sorafenib was the first multityrosine-kinase inhibitor, blocking different receptors, including Raf, the vascular endothelial growth factor, and platelet-derived growth factor, expressed by signaling pathways in HCC. Considering its large approval worldwide, sorafenib was employed not only for patients in an advanced stage of the disease but also as a bridging therapy to downstage the disease and include patients in the transplantation list[49].

Currently, the clinical landscape for patients with advanced liver cancer has changed quickly. Different agents were approved for clinical use, including lavatinib, cabozantinib, regorafenib, and ramucirumab, all addressed to the aforementioned pathways[50]. Moreover, different signs of progress have been made in immunotherapy, in particular with the advent of immune check-point blockers. Nivolumab (anti-PD-1 antibody), pembrolizumab (anti-PD-1 antibody), tremelimumab (anti-CTLA-4 antibody), and atezolizumab (anti-PD-L1 antibody) were tested for advanced HCC[51].

In 2022, Reig *et al*[10] refreshed the BCLC strategy for prognosis prediction and treatment recommendations. It has been established that the first line treatment of advanced HCC should be based on a combined approach. Atezolizumab with bevacizumab (anti-vascular endothelial growth factor antibody) is currently the first-choice first-line treatment. Finn *et al*[52], in a global, open-label, phase 3 trial, demonstrated the best OS and PFS of the combined therapy in comparison with sorafenib alone. Conversely, the atezolizumab-bevacizumab treatment can be used in patients with compensated Child-Pugh A cirrhosis and risk of upper gastrointestinal bleeding.

The second-line treatment is not well established yet. If patients underwent sorafenib treatment, then it is possible to evaluate the benefit from regorafenib[53], cabozatinib[54], or ramucirumab[55]. If the second-line treatment cannot add a clinical benefit or is not feasible due to patient contraindications, then the third-line treatment with cabozatinib can be considered to increase OS[56]. Finally, if all previously mentioned cases are not manageable, patients should be enrolled in clinical trials. Clinical and laboratory data used to choose the preferred medical treatment are out of the scope of the present review.

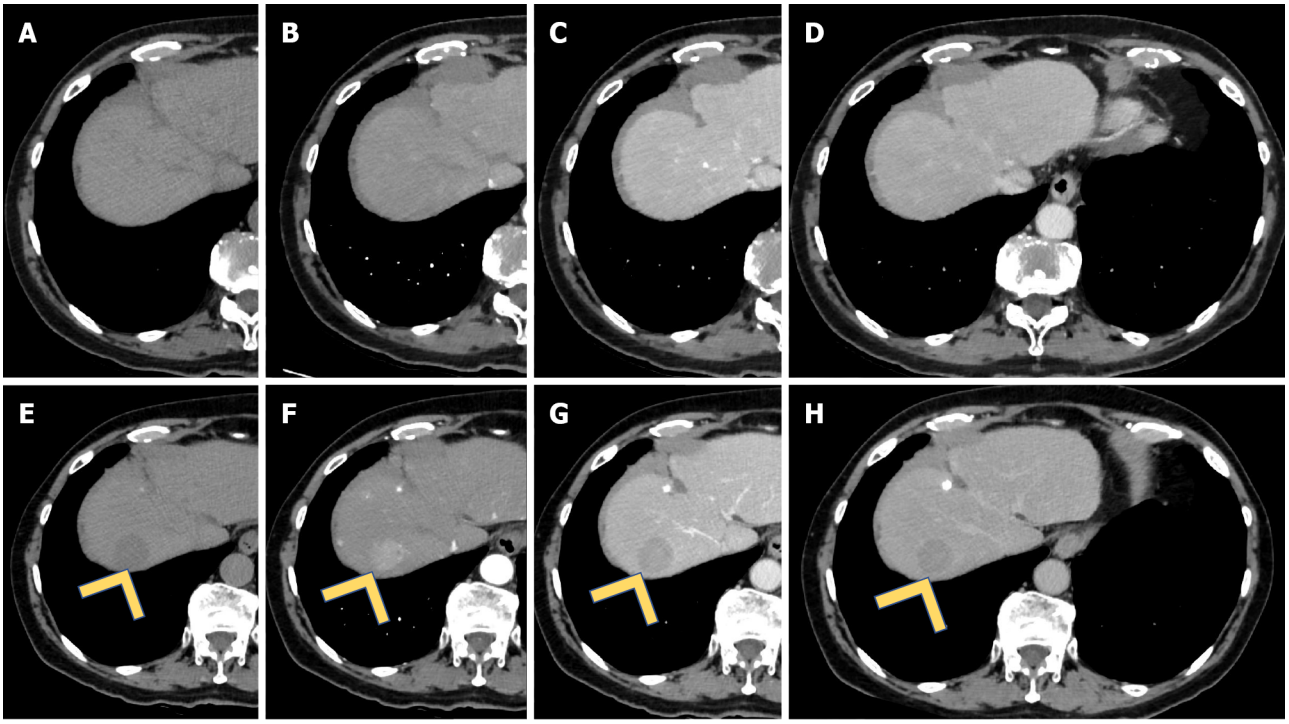


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Figure 3 Magnetic resonance imaging follow-up study with GD-EOB-DTPA for assessment of treatment response (after transarterial chemoembolization and radiofrequency ablation). A and B: A 70-year-old female underwent conventional transarterial chemoembolization-radiofrequency ablation of a hepatocellular carcinoma lesion located in the VIII hepatic segment. Eighteen months after treatment, confluent areas of hyperintense signal on T2 weighted imaging, with and without fat saturation, represented fibrosis. In this context a small slightly hyperintense nodular lesion was seen on T2 weighted imaging (T2 and T2 fs, yellow arrows); C-F: This lesion was isointense to the liver parenchyma in the unenhanced phase (C), with a non-peripheral wash-in appearance during the arterial phase (D), isointense during the portal venous phase (E), and hypointense during the hepatobiliary phase acquired after 20 min of Gd-EOB-DTPA administration (F). The final diagnosis was recurrent hepatocellular carcinoma after transarterial chemoembolization-radiofrequency ablation.

In this setting, patients who underwent LRTs should be followed up due to the risk of recurrence. In patients who underwent medical approaches it is important to monitor tumor response. All the above-mentioned medical strategies can determine apoptosis or necrosis of tumoral cells. One of the most important common findings to evaluate during follow-up is the change in tumor size. A significant increase in tumor volume or maximum axial diameter should be considered as a progression, according to the World Health Organization (WHO) criteria[56]. However, over time, different clinical studies were focused on the main issues related to the WHO classification. Consequently, RECIST 1.1 was introduced in clinical practice. However, RECIST 1.1 has some limitations, including the increase or decrease in size and necrosis, not being taken into account[57]. This last aspect is extremely important during medical treatments since the majority of drugs employed for HCC induce a reduction in tumor vascularization. For these reasons it is important to acquire images with complete protocols, to detect typical radiological findings of the primitive tumor, and to collect every significant change. First, increased dimensions of hypervascular areas or nodules should be considered as a main finding of tumor recurrence or progression[58,59]. To evaluate these, it is of utmost importance to acquire a correct arterial phase both on computed tomography (CT) and magnetic resonance imaging (MRI).

In 2014, Salvaggio *et al*[60] aimed to collect HCC enhancement changes after sorafenib treatment. The authors demonstrated that after medical treatment both arterial and portal venous enhancement was significantly reduced. In particular, the authors demonstrated that patients with partial response can manifest a greater decrease in arterial phase enhancement. However, they did not demonstrate the opposite. Patients with progressive disease did not show any statistically significant difference in arterial phase enhancement before and after treatment. To better understand the medical response, the international literature moved to the usefulness of MRI. Choi *et al*[61] reviewed the most common imaging findings of HCC during medical treatment by using MRI. The authors reported the importance of the hypervascular appearance during the arterial phase, as reported for CT. Moreover, MRI can help to detect early responders from non-responders by using diffusion-weighted imaging (DWI) and apparent diffusion coefficient maps, showing in the first group of patients an increase of DWI signal



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Figure 4 Multiphasic computed tomography study for assessment of treatment response (after sorafenib). A-D: A 66-year-old female underwent conventional medical therapy (sorafenib) for an advanced hepatocellular carcinoma (HCC) lesion. Computed tomography images represented the complete response to the medical therapy, with no areas or nodular lesions suspected for HCC; E-H: During the follow-up, 2 years after completion of therapy, a nodular hypoattenuating lesion in the unenhanced phase (E) appeared in the VII hepatic segment. This lesion had similar features of primary HCC, with non-rim hyperenhancement during the arterial phase (F), wash-out during the portal venous phase (G) and delayed phase (H). This is an example of HCC recurrence after sorafenib.

with correspondence on apparent diffusion coefficient map due to necrosis and reduced tumor cellularity. Finally, MR can benefit from the usefulness of hepatobiliary contrast agents, as demonstrated in the SORAMIC trial[62]. However, by searching PubMed and EMBASE no important studies have been published yet about this promising added value, and future studies should be focused on these aspects.

The advent of all the above-mentioned strategies, alone or combined, introduced a new class of response[52]. While about 8% can show a hyperprogression, a new atypical response is included in the iRECIST criteria[63]. However, no predictive biomarkers can help clinicians to determine the risk of atypical response during immunotherapy, and only the radiological approach, both with CT and MRI, can help follow patients during the treatment. Even if in the past medical treatment was considered the last useful medical treatment in advanced HCC, different ongoing studies are testing a combination of only medical drugs and in combination with LRTs, such as TACE, as reported by Pinter *et al*[64].

Combined strategies may be useful in advanced RHCC. Peng *et al*[65] showed that sorafenib combined with TACE-RFA was superior to therapy with sorafenib alone concerning time to progression and OS in patients with RHCC with one intrahepatic tumor size ≤ 7 cm or ≤ 5 cm intrahepatic nodules, with each tumor ≤ 3 cm.

RADIOLOGICAL APPROACH TO RHCC

Radiology plays a central role in the assessment of patient response LRT for RHCC. The identification of viable tumor treatment guides for further management, and it potentially affects transplantation eligibility. In these instances, it is often helpful to engage in a multidisciplinary discussion to determine how to best manage each patient. The Liver Imaging Reporting and Data System (LI-RADS) was developed in 2011 to relay the likelihood of HCC on CT or MRI in a standardized manner, in patients at risk for HCC. In 2017, the LI-RADS treatment response algorithm (LI-RADS TRA) was introduced for the assessment of lesions that have been previously treated with LRT[66]. Unlike the prior response criteria RECIST and WHO that focus on disease progression on a systemic level, LI-RADS TRA is based on enhancement features to predict viability on a lesion level[67]. Although modified RECIST (mRECIST) has historically been used for the evaluation of HCC after locoregional therapy, differences from LI-RADS TRA include a lack of equivocal category and a lack of additional features for diagnosing tumor viability[68]. mRECIST uses the presence of arterial enhancing components alone to diagnose

viability while LI-RADS TRA includes additional imaging features such as washout during the portal venous or delayed phases and enhancement similar to pre-treatment to define viable tumors and encompass the equivocal category in addition to the binary evaluation[69].

RECOMMENDATIONS FOR HCC RECURRENCE DETECTION

Non-invasive imaging is superior to any other method for the surveillance of patients at risk of developing RHCC, either after OLT or other curative treatments. However, robust data lacks the optimal follow-up schedule of HCC-treated patients. Notably, international guidelines slightly differ in the recommended follow-up intervals, ranging from 3 mo to 6 mo, and duration of cross-sectional imaging after curative treatments. The National Comprehensive Cancer Network panel recommends ongoing total-body surveillance with multiphasic cross-sectional imaging (*i.e.* CT or MRI) every 3 mo to 6 mo for 2 years, then every 6 mo to 12 mo after curative therapies[70]. The 2018 Practice Guidance by the American Association for the Study of Liver Diseases suggests surveillance for HCC recurrence in posttransplant patients with abdominal and chest CT scan, though timing and duration as well as the impact of surveillance are not univocally defined[71]. After ablative therapies, the American Association for the Study of Liver Diseases recommends surveillance with contrast-enhanced CT or MRI every 3-6 mo[71].

The 2018 European Society for Medical Oncology Clinical Practice Guidelines were endorsed by the pan-Asian consensus conference, which included experts from several Asian societies. However, the Asian-adapted version slightly changed the follow-up timing after curative treatment, limiting the 3-mo interval by dynamic CT or MRI studies to the 1st year instead of 2[72,73]. Also, the European Association for the Study of the Liver recommends a follow-up after resection with curative intent with 3-4 mo intervals limited to the 1st year after treatment, with a return to regular surveillance thereafter[4].

Interestingly, Kim *et al*[74] found that HCC patients who undergo curative treatments with complete response and who present with increasing alpha-fetoprotein levels have a high probability of impending tumor recurrence even in the presence of a negative MRI. The follow-up schedule proposed within the European Society for Medical Oncology guidelines for patients treated with TACE or systemic therapies includes contrast-enhanced CT or MRI every 3 mo[74].

All the above-mentioned guidelines converge on the equivalent role of CT and MRI in clinical practice, given that the most important aspect for the diagnosis of HCC is the definition of criteria with the highest achievable accuracy, regardless of the imaging technique. Erkan *et al*[75] reviewed 3491 pathologically examined liver lesions, either studied by CT or MRI, comparing the diagnostic performance of different non-invasive diagnostic criteria of HCC. They found no statistically significant differences among criteria in diagnostic accuracy, with LI-RADS performing the best in terms of sensitivity and accuracy. Nevertheless, though CT and MRI have comparable performance in clinical practice, they present specific features to be considered.

CT

CT has the advantage of being the most practical and widely available tool to perform surveillance in HCC-treated patients. Its main limitations consist of ionizing radiation exposure and iodinated contrast agents-related nephrotoxicity. The detection and characterization of liver nodules with conventional contrast-enhanced CT is substantially limited to the size, morphology, and enhancement pattern of the lesions, which are sufficient elements to reach a confident diagnosis according to LI-RADS. RHCC imaging findings are analogous to the primary lesion. In particular, the typical hallmarks in the imaging diagnosis of RHCC are the combination of hyperenhancement in the arterial phase and washout on the portal venous or delayed phases[4]. Several studies and meta-analyses have compared the performance of CT with other imaging techniques. In a multicenter prospective trial including 544 nodules in 381 patients, the sensitivity and specificity for the diagnosis of 10-20 mm HCC nodules were 67.9% and 76.8%, respectively, while for the 20-30 mm HCC nodules, the sensitivity and specificity were higher (71.6% and 93.6%, respectively)[76]. In a meta-analysis, CT had an overall sensitivity of 72% with a subgroup analysis revealing a sensitivity of 31% *vs* 82% for sub-centimetric lesions compared to ≥ 1 cm ones[77]. Of note, this data did not consider the prevalence of HCC diagnosis in HCC-naïve patients compared to previously treated patients, for whom the pre-test probability of disease is expected to be increased. A multicenter prospective study that enrolled patients scheduled for liver imaging before surgery showed a sensitivity of 70%[78].

MRI

The accuracy of MRI in detecting HCC, especially small nodules, is superior to that of CT as shown by several studies and meta-analyses, one of which reported a sensitivity of 82% compared with 66% of CT and a comparable specificity[4,79]. However, MRI is yet to be definitively recommended over CT, given that the quality of the available evidence is considered low[79]. Moreover, a distinction between extracellular contrast agents (ECA) and hepatobiliary contrast agents (HBCA) should be considered. Analogous to those used in CT, ECA detects and characterizes lesions through the enhancement pattern.

Conversely, HBCA provides information on the hepatocellular function and bile excretion. Typical nodule hypointensity against a strongly enhanced background parenchyma in the hepatobiliary phase increases RHCC conspicuity and delineation, facilitating detection and consequently the diagnosis[80]. Despite this advantage, it must be pointed out that, if considered alone, hepatobiliary phase imaging is non-specific. Therefore, it always requires interpretation together with the dynamic study[81]. Martino *et al*[82] reported significantly higher diagnostic accuracy, sensitivity, and negative predictive value when dynamic and hepatobiliary phase MRI were combined compared to CT and dynamic phase MRI alone; a particular diagnostic benefit was obtained for lesions between 1 cm and 2 cm.

Nevertheless, although most HCC lesions are typically hypointense during the hepatobiliary phase, about 5%-12% HCC lesions can be hyperintense, owing to the overexpression of OATP[81]; conversely, some benign nodules may show no contrast uptake[83]. The knowledge of the pathological features of the originally treated nodules may predict the behavior of recurrent disease on hepatobiliary phase imaging, improving diagnostic confidence.

Different HBCA molecules have specific pharmacokinetic profiles. Gadoxetate disodium presents a 50% hepatic excretion, which contributes to an early liver parenchyma enhancement. Conversely, gadobenate-dimeglumine has a 3%-5% hepatic excretion that delays the hepatobiliary phase imaging onset. As a consequence, gadoxetate disodium does not provide a conventional delayed vascular phase but instead shows a transitional phase that lasts for several minutes, representing a transition from extracellular-dominant to intracellular-dominant enhancement[81]. Interestingly, Yim *et al*[84] recently observed that, in a retrospective cohort of patients who underwent both ECA and HBCA, RHCC was diagnosticated with higher accuracy using ECA.

DWI has been shown to improve the accuracy of RHCC detection, especially when combined with gadoxetic acid-enhanced imaging[85,86]. Finally, a recent meta-analysis confirmed that DWI may improve the ability to detect residual HCC or RHCC after TACE[87].

MRI likely has the highest accuracy compared to other imaging techniques in the detection of small recurrence after curative treatments[43]. However, results interpretation according to the standard LI-RADS may suffer from reduced sensitivity and specificity for disease recurrence detection. Wang *et al*[88] found that non-rim arterial phase hyperenhancement and three ancillary features (hepatobiliary phase hypointensity, mild-moderate T2 hyperintensity, and restriction of diffusion) were significantly related to RHCCs < 20 mm and concluded that the characterization of < 10 mm recurrence may show improved specificity compared with the LI-RADS 4 category combining at least two ancillary features. However, in patients treated with systemic therapies, according to the mRECIST criteria, new HCC lesions must measure at least 1 cm to define disease progression[58]. Despite the high sensitivity of MRI to detect recurrence after curative treatments, it has been shown that small viable RHCC may hide behind false-negative studies. This warrants regular short-term imaging surveillance[89].

However, in the absence of evidence to recommend a particular method or contrast agent over the other, practitioners are encouraged to base the choice on their judgment on an individual basis, considering the local availability of resources, personal experience, and imaging features of the previously-treated HCC[71].

Contrast-enhanced US

The use of contrast-enhanced US (CEUS) is encouraged as it has been demonstrated that its specificity can be even superior compared to CT/MRI[76]. Although CEUS is inferior to both CT and MRI in terms of objectivity and panoramic view, it provides advantages in cases of renal dysfunction and iodine allergy. The current indications for CEUS are multiple, the most important of which are equivocal or inconclusive findings on CT or MRI studies and assessment of treatment response after TACE or ablation[90]. Bansal *et al*[91] proposed an algorithm with alternating MRI and CEUS for secondary surveillance following potentially curative therapy of HCC. In their prospective studies, the authors found similar diagnostic performance of the two techniques; of note, CEUS was able to confirm or disprove equivocal findings on MRI. The comparable diagnostic performance of CEUS, CT, and MRI was previously reported[92].

It has been reported that RHCC may differ from the initial tumor at imaging, and this may help to distinguish recurrence form residual diseases, which may have a prognostic relevance. Wu *et al*[93] recently described different CEUS patterns of RHCC compared to initial tumors: Among the others, more homogeneous enhancement, poorly defined borders, and marked washout were found to be typical features of recurrent disease.

The application of artificial intelligence and radiomics to preoperative CEUS has recently gained large interest, and it has been demonstrated to potentially predict the prognosis in terms of HCC recurrence and overall survival[94-97]. CEUS, added to other conventional US-based techniques, has also shown the ability to improve the prediction of microvascular invasion, which is probably the most important factor associated with a worse prognosis[98]. Finally, CEUS can be useful as guidance for ablative therapies, especially to target recurrence of previously treated lesions[99,100].

Perfusion CT/MRI

Perfusion imaging does not have a definite role in clinical practice, and it is mainly performed for investigative purposes. Although several authors have independently demonstrated that perfusion CT-

derived parameters can discriminate between normal liver parenchyma, HCC, and hypervascular pseudolesions[101-103], they are yet to be included in clinical practice guidelines due to the absence of standardization among different centers[104]. However, perfusion imaging that provides quantitative parameters that could potentially be more reliable than qualitative/subjective parameters seems promising in the assessment of tumor response both to locoregional and systemic therapies[105-114].

Compared to CT, perfusion MRI has been investigated more regarding the possibility of predicting microvascular invasion of HCC before treatment. The microvascular invasion has been demonstrated to be correlated with poor outcomes of curative therapies due to higher rates of disease recurrence[115]. Perfusion MRI can be performed either with dynamic contrast-enhanced studies or with the intravoxel incoherent motion diffusion-weighted technique[116-119].

Nuclear medicine

The role of nuclear medicine in the diagnosis and staging of HCC is debated. If on the one hand there is insufficient evidence to recommend the use of fluorodeoxyglucose positron emission tomography preoperatively, it has been demonstrated that nuclear medicine studies are able to predict tumor aggressiveness and may aid in identifying those patients at risk for HCC recurrence after liver transplantation, resection, or ablation for better treatment allocation[120,121]. Fluorodeoxyglucose positron emission tomography, with or without CT, has also been shown to present low sensitivity but high specificity for diagnosing extrahepatic metastases or local residual/recurrent HCC after treatment [121].

CONCLUSION

On the one hand, the assessment of the response to LRTs has been widely described[121-123]. On the other hand, histologic modifications induced by molecular therapies may explain different imaging findings of recurrent disease. Differentiation between treatment-induced tumor necrosis and viable tumor with reduced arterial perfusion may be challenging. After treatment with systemic targeted therapy, the tumor may show areas of necrosis without any contrast enhancement that must be distinguished from areas of reduced but still unequivocal arterial uptake consistent with viable tumor[44]. Even RHCC under systemic treatments may present with atypical enhancing patterns, especially lacking arterial hyperenhancement, which makes radiological assessment more difficult. All these aspects should be considered, and multimodal imaging evaluation combined with multidisciplinary framework can improve image interpretation. Conventional non-invasive imaging techniques provide robust criteria for HCC residual/recurrence detection, with high accuracy, representing the current standard of practice. Advanced imaging tools, either hardware- or software-based, have a double potential role: to predict HCC treatment response or the risk of recurrence, to increase sensitivity, specificity, and thus operator confidence in early RHCC detection.

FOOTNOTES

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REVIEW

Factors affecting the quality of bowel preparation for colonoscopy in hard-to-prepare patients: Evidence from the literature

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Abstract

Adequate bowel cleansing is critical for a high-quality colonoscopy because it affects diagnostic accuracy and adenoma detection. Nevertheless, almost a quarter of procedures are still carried out with suboptimal preparation, resulting in longer procedure times, higher risk of complications, and higher likelihood of missing lesions. Current guidelines recommend high-volume or low-volume polyethylene glycol (PEG)/non-PEG-based split-dose regimens. In patients who have had insufficient bowel cleansing, the colonoscopy should be repeated the same day or the next day with additional bowel cleansing as a salvage option. A strategy that includes a prolonged low-fiber diet, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may increase cleansing success rates in the elderly. Furthermore, even though no specific product is specifically recommended in the other cases for difficult-to-prepare patients, clinical evidence suggests that 1-L PEG plus ascorbic acid preparation are associated with higher cleansing success in hospitalized and inflammatory bowel disease patients. Patients with severe renal insufficiency (creatinine clearance < 30 mL/min) should be prepared with isotonic high volume PEG solutions. Few data on

cirrhotic patients are currently available, and no trials have been conducted in this population. An accurate characterization of procedural and patient variables may lead to a more personalized approach to bowel preparation, especially in patients undergoing resection of left colon lesions, where intestinal preparation has a poor outcome. The purpose of this review was to summarize the evidence on the risk factors influencing the quality of bowel cleansing in difficult-to-prepare patients, as well as strategies to improve colonoscopy preparation in these patients.

Key Words: Colorectal cancer; Colonoscopy; Adenoma detection rate; Bowel preparation; Polyethylene glycol

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Core Tip: Almost a quarter of procedures are still performed with inadequate preparation. A strategy that includes a low-fiber diet for an extended period of time, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may improve cleansing success rates in the elderly. In addition, while no specific product is recommended for difficult-to-prepare patients, clinical evidence suggests that 1-L polyethylene glycol (PEG) plus ascorbic acid preparation is associated with higher cleansing success in hospitalized and inflammatory bowel disease patients. Isotonic high volume PEG solutions should be given to patients with severe renal failure.

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INTRODUCTION

One of the most commonly used techniques for diagnosing colorectal diseases is colonoscopy. Furthermore, it is important in colorectal cancer (CRC) screening because early detection is linked to a long-term reduction in malignancy incidence and mortality[1,2].

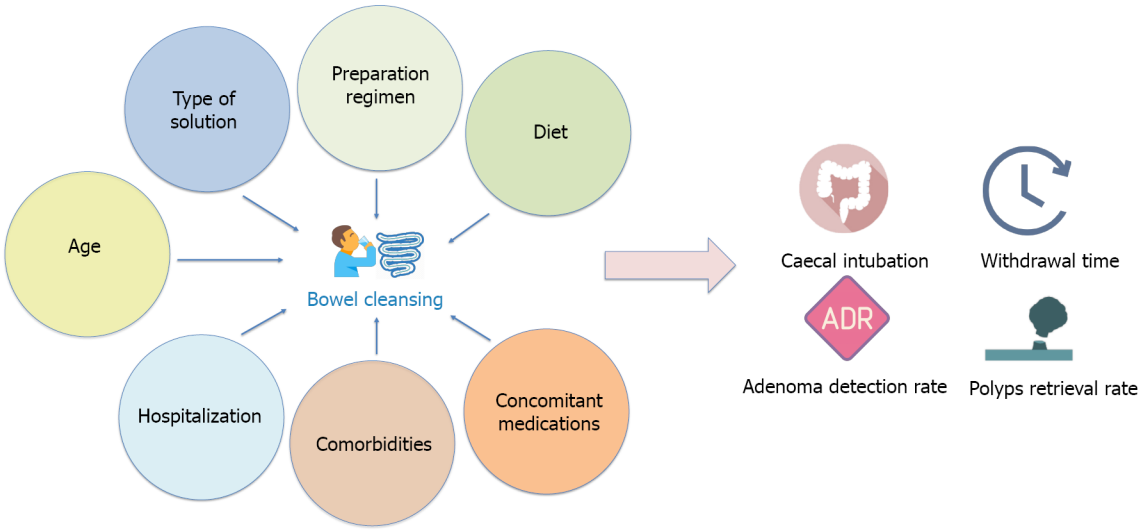
As is well known, the quality of a colonoscopy is entirely dependent on adequate bowel cleansing, which can affect diagnostic accuracy and the rate of adenoma detection (ADR)[3]. Inadequate bowel preparation, on the other hand, leads to decreased sensitivity to colonoscopy, increased procedural time, a higher risk of adverse events, and a greater likelihood of having to repeat the exam at a higher cost[4-7].

This is a critical topic because data from the literature show that a quarter of procedures still have suboptimal preparation[8]. Similarly, a large Italian study discovered that poor preparation occurs in approximately 17% of colonoscopies[9], and results from the United Kingdom screening program confirmed that inadequate preparation accounts for more than 20% of incomplete procedures[10]. Finally, data from a recent survey of sixty-four Italian screening centers revealed that only 29% of centers meet the minimum standard of at least 90% colonoscopies with adequate cleansing[11].

The type of bowel preparation, split-dose regimen, low-fiber diet, comorbidities, concomitant medications, inpatient status, and elderly age have all been found to affect the quality of bowel cleansing (Figure 1)[12]. Some of these variables, such as the type of solution, preparation regimen, and diet, are modifiable risk factors. In this regard, recent scientific advances have resulted in the introduction of new effective bowel cleansing solutions and reinforced the importance of specific preparation regimens.

On the other side, factors such as age or comorbidities are not modifiable and not susceptible to intervention. This makes it more difficult to intervene to increase the quality of bowel cleansing in specific conditions, also considering that guidelines do not provide specific recommendations for patients with multiple risk factors[13].

The purpose of this review is to summarize the evidence on the risk factors that influence the quality of cleansing in difficult-to-prepare patients, as well as strategies to improve colonoscopy preparation in these patients.



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Figure 1 Factors affecting bowel preparation and impact on colonoscopy outcomes.

FACTORS INFLUENCING THE QUALITY OF BOWEL PREPARATION

Type of solution and tolerability

Several laxatives are currently available and have been studied, with varying efficacy and tolerability.

Among these solutions are polyethylene glycol plus ascorbate (PEG-ASC) (4-L, 2-L, and 1-L), 2-L PEG plus citrate, 2-L PEG plus Bisacodyl, magnesium citrate plus picosulphate (MCSP), and trisulfate (magnesium sulfate, sodium sulfate, and potassium sulfate), also known as oral sulfate solution (OSS).

A meta-analysis of PEG solutions discovered that split-dose high-volume PEG was more effective than other options, including low-volume regimens [odds ratio (OR) = 3.46, 95% confidence interval (95%CI) 2.45-4.89][14]. Another meta-analysis comparing split-dose high-volume PEG to split-dose low-volume PEG found that high-volume regimens were superior (OR = 1.89, 95%CI 1.01-3.46)[15]. Nonetheless, because high-volume solutions may reduce patient compliance, resulting in suboptimal preparation, new low-volume laxatives have been introduced in the last decade.

Following that, a 2-L PEG plus citrate and simethicone solution was added. Randomized controlled trials (RCTs) comparing this formulation 2-L PEG plus citrate *vs* 4-L PEG found similar cleansing efficacy (73.6% *vs* 72.3%, 95%CI difference -7.5 to 10.1), but with greater tolerability (25.4% *vs* 37.0%, $P <$ 0.01) and acceptability (93.9% *vs* 82.2%, $P < 0.001$)[16].

Moreover, 2-L PEG plus citrate showed similar efficacy (78.3% *vs* 74.3%, $P = 0.37$) and acceptability (81.4% *vs* 80.8%, $P = 0.74$) also when compared to 2-L PEG-ASC[17].

Recently, a 1-L PEG-ASC solution with a very low volume has been introduced to improve patients' experience during colonoscopy by reducing the total oral intake of liquids to be consumed. The development of this very low-volume solution was made possible by increasing the ascorbate content, which improves the laxative effect and allows the solution to be delivered in a smaller volume.

Three phase-3 RCTs comparing the effectiveness of 1-L PEG-ASC (Plenvu, Norgine, Harefield, United Kingdom) *vs* trisulfate[18], sodium picosulfate plus magnesium citrate[19], and 2-L PEG[20] revealed that 1-L PEG-ASC was non-inferior to the comparator.

Moreover, a multicenter prospective study was performed to assess the effectiveness of 1-L PEG-ASC compared to 2-L and 4-L PEG preparation in a real-life setting on a cohort of 1289 patients ($n = 490$ performing a 4-L PEG preparation, $n = 566$ a 2-L PEG and $n = 233$ a 1-L PEG)[21].

In this study, cleansing success was achieved in 72.4%, 74.1%, and 90.1% ($P < 0.001$), respectively, while high-quality cleansing of the right colon was achieved in 15.9%, 12.0%, and 41.4% ($P < 0.001$) for the 4-L, 2-L, and 1-L-PEG preparation groups, respectively. The 1-L PEG-ASC preparation was an independent predictor of overall cleansing success and high-quality cleansing of the right colon in multiple regression analysis. In that study, 44.8% of patients were over the age of 65, confirming the validity of the safety of 1-L PEG-ASC in the elderly.

A real-life study performed on a cohort of hospitalized subjects confirmed that, among all variables, the 1-L PEG-ASC solution (OR = 0.39, 95%CI 0.23-0.65) and a > 75% intake of bowel preparation (OR = 0.09, 95%CI 0.05-0.15) significantly reduced the risk of inadequate colon cleansing[22].

This information is undoubtedly valuable and should be considered even in the care of hospitalized patients.

Nonetheless, the same studies mentioned above found that 1-L PEG-ASC is associated with a higher incidence of adverse events, such as nausea and vomiting, even if none of these were serious enough to

impair bowel cleansing quality[18-21].

Based on current data, guidelines recommend using high-volume or low-volume PEG-based regimens, and non-PEG-based validated products, in a split-dose fashion[13].

Even though no specific product is recommended for difficult-to-prepare patients, 1-L PEG-ASC appears to be promisingly superior in hospitalized patients and could be a reasonable choice in this setting; however, these data were obtained after the guidelines were issued.

Preparation regimen

The preparation regimen has a significant impact on the quality of bowel cleansing. A day before preparation was traditionally performed before a colonoscopy. Nonetheless, the most recent evidence suggests that dividing the preparation between the day before the exam and the day of the exam leads to better intestinal cleansing and a shorter time between the end of the preparation and the same colonoscopy.

A meta-analysis of 47 trials involving 13487 patients revealed that a split-dose regimen is associated with significantly better cleansing than day-before preparations (OR = 2.51, 95%CI 1.86-3.39), regardless of solution type and dose[15].

Furthermore, subgroup analysis by solution type confirmed that split was more effective than the day-before regimen for PEG, sodium phosphate, and picosulfate (OR = 2.60, 95%CI 1.46-4.63; OR = 9.34, 95%CI 2.12-41.11; OR = 3.54, 95%CI 1.95-6.45, respectively). Likewise, a higher proportion of patients (OR = 1.90, 95%CI 1.05-3.46) were willing to repeat preparation with split-dose *vs* day-before cleansing [15].

Subsequent RCTs evaluating the effectiveness of high-volume PEG[23,24], low-volume PEG[25,26], and sodium picosulfate[27-29] confirmed the split regimen's superiority over the day-before regimen.

In terms of the effectiveness of a split regimen on the detection of neoplastic lesions, an RCT comparing 2-L PEG-ASC in a split *vs* day-before fashion found that a split regimen resulted in a higher detection rate of adenomas (53.0% *vs* 40.9%; RR = 1.22, 95%CI 1.03-1.46) and advanced adenomas (26.4% *vs* 20.0%; RR = 1.35, 95%CI 1.06-1.73)[26].

Another RCT comparing 2-L PEG-ASC in split dose *vs* split-dose sodium picosulfate/magnesium citrate (SPMC) in a day-before dose showed a non-significant higher polyp detection rate, and a significantly higher detection rate of right-sided polyps and adenomas (51.5% *vs* 44.0%, $P = 0.14$; 28.0% *vs* 16.6%, $P = 0.007$; 21.0% *vs* 11.9%, $P = 0.015$, respectively) in favor of split regimen[30].

Also, two meta-analyses (including 11 and 14 RCTs) compared split-dose bowel preparation with same-day bowel preparation and found similar results in terms of bowel preparation quality, patient willingness to repeat it, and overall tolerability[31,32].

In addition to the split preparation, patients who need to have a colonoscopy in the afternoon should consider same-day preparation. In this regard, data from two meta-analyses revealed that when split and same-day regimens were used, the quality of bowel preparation, tolerability, and willingness to repeat were similar.

As a result, for patients undergoing afternoon colonoscopy, current guidelines recommend split-dose bowel preparation and same-day bowel preparation[13].

Diet before the procedure

Before a colonoscopy, a low-residue diet or clear liquids are usually advised. Patients should avoid foods high in fiber, such as fruits, vegetables, and whole grains, to achieve a low-residue diet. Colored liquids should be avoided because they can obscure proper mucosal visualization. Water, broth, coffee or tea, ice, gelatin, and fruit juices are the best clear liquid choices (apple, lemonade, and grapefruit).

On the day before the colonoscopy, two meta-analyses compared a low-residue diet to a clear liquid diet, with both arms using the same laxative. When compared to a clear liquid diet, Nguyen *et al*[33] discovered that a low residue diet was associated with better tolerability (OR = 1.92, 95%CI 1.36-2.70, $P < 0.01$) and higher willingness to repeat bowel preparation (OR = 1.86, 95%CI 1.34-2.59, $P < 0.01$). However, no differences in adequate bowel cleansing (OR = 1.21, 95%CI 0.64-2.28, $P = 0.58$) or adverse event incidence (OR = 0.88, 95%CI 0.58-1.35, $P = 0.57$) were found[33]. Another meta-analysis, this time by Avalos *et al*[34], compared a low-residue diet or a regular diet to a clear liquid diet. There were no differences in adequate cleansing (RR = 1.00, 95%CI 0.97-1.04). The low residue-regular diet was linked to a higher likelihood of repeating the procedure, better tolerability, and more frequent consumption of the solution (RR = 1.08, 95%CI 1.01-1.16; RR = 1.04, 95%CI 1.01-1.08; RR = 1.04, 95%CI 1.01-1.08, respectively). Except for more hungriness in the clear liquid diet group, there were no differences in ADR or adverse events between groups (RR = 1.93, 95%CI 1.13-3.30)[34]. In a third meta-analysis by Song *et al*[35] including 7 RCTs using different laxatives, a low residue diet showed higher tolerability (RR = 1.06, 95 %CI 1.02-1.11) and a higher likelihood of repeating the exam (RR = 1.17, 95%CI 1.09-1.26) compared with a clear liquid diet.

Concerning the duration of the diet, current guidelines recommend a low-residue diet or clear liquids for at least one day before the examination[13]. In this regard, two recent RCTs compared the 1-d *vs* 3-d diet, finding no significant difference after diet prolongation. The first RCT compared a 3-d *vs* 1-d low-fiber diet with a 4-L PEG split-dose preparation and found a similar rate of adequate bowel cleansing (91.7% for 3-d diet *vs* 94.7% for 1-d diet, $P = 0.24$)[36]. Similarly, the second RCT compared a 1-d *vs* 3-d

low-residue diet with a 2-L PEG + ASC split-dose preparation (82.7% *vs* 85.6%), with no differences in adherence, satisfaction, or polyp or ADR[37].

However, it should be noted that fiber consumption varies greatly between people and regions of the world. As a result, in countries where fiber consumption is naturally low, a single day's diet is more likely to suffice. On the contrary, in people or countries where fiber consumption is abundant, the diet should be tailored accordingly, particularly in hard-to-prepare patients.

Timing of colonoscopy after bowel preparation

The timing of colon preparation administration has a significant impact on both its tolerability and efficacy. Starting the preparation, the day before the colonoscopy improves the quality of colonic cleansing and the likelihood of finding flat lesions.

According to recent research, there is a negative correlation between the start of the colonoscopy, the interval since the last dose of bowel preparation, and the cleanliness of the mucosa. It is recommended to perform the colonoscopy sooner after the bowel preparation is complete to improve the quality of the examination[38,39].

Patients with intervals of 7 h or less between the start of PEG intake and the start of the colonoscopy had better bowel preparation than patients with intervals of more than 7 h (P = 0.03)[40].

Furthermore, bowel cleansing with the fractional regimen was superior within three hours of the last dose intake, declined gradually after 4-5 h, and became statistically insignificant at five hours, according to Bucci *et al*'s meta-analysis in 2014 including 29 RCTs comparing split *vs* day-before regimens[41].

Additionally, a subsequent multicenter, randomized, endoscopist-blinded study found that the best window of time for bowel preparation is the same for all preparations. Regardless of the preparation (PEG, PEG-ASC, ≤ 11.8, sodium picosulphate/magnesium citrate, ≤ 13.3 h), the ideal time before the colonoscopy was ≤ 11.8 h. The timing of the preparation did not affect the tolerability[42].

Anyway, the overall findings support the recommendation in the European Society of Gastrointestinal Endoscopy (ESGE) guidelines to begin the final dose of bowel preparation no later than 5 h before the colonoscopy and to finish it no later than 2 h before the procedure[13].

Of note, compared to other days of the week, Monday had the highest rate of inadequate preparation [Boston bowel preparation scale (BBPS) 6] of 16.5% in a 2022 retrospective review of 4279 colonoscopies. Notably, poor bowel preparation was not linked to post-holiday procedures[43].

However, in clinical practice, patients do not always adhere to the recommended timing due to a need related to the potential long distance to travel before having to perform the endoscopic exam, or as a result of the stress that they will have an urgent necessity for the public bathroom during the trip.

As a result of more accurate characterization of procedural and patient variables, bowel preparation may become more personalized.

SPECIFIC CLINICAL SETTINGS

Hospitalized patients

Hospitalization is associated with a nearly twofold increase in the risk of unsuccessful bowel preparation before colonoscopy when compared to the ambulatory setting[44,45].

Furthermore, the percentage of inpatients with an adequately prepared colon does not exceed 50% because they are typically elderly, frail, and suffering from comorbidities that either prevent successful bowel prep ingestion or affect patients' comprehension and compliance with the regimen's instructions [44,45].

Inadequate bowel preparation increases the risk of missed adenomatous polyps or CRC, as well as a patient inconvenience; it also harms healthcare systems by causing procedures to be delayed or repeated, as well as prolonged hospital stays[45-47].

Several studies have evaluated the efficacy of various interventions, such as different laxatives, changes in preparation administration timing, and promotion of educational programs for physicians, nurses, and patients, to overcome these challenges. Lower socioeconomic class, opiate/tricyclic antidepressant use, afternoon colonoscopies, American Society of Anaesthesiologists (ASA) physical status classification system class 3, and pre-preparation nausea/vomiting were identified as the main potential predictors of inadequate inpatient preparation[46].

Furthermore, Gkolfakis *et al*[45] recently published a systematic review with meta-analysis to evaluate the efficacy of various interventions to improve the quality of colon preparation in inpatients.

In this study, which included 17 studies and 2733 inpatients, the authors concluded that, despite several interventions, only nearly two-thirds of inpatients achieve adequate colon preparation before colonoscopy, and that educational interventions significantly improve inpatients' bowel preparation quality[45].

In the absence of standardized guidelines or recommendations, the ideal bowel preparation regimen for inpatients has yet to be determined[45].

In any preparation strategy, PEG-based regimens should be considered first because they are more likely to achieve adequate bowel cleansing while maintaining an optimal patient safety profile. Furthermore, case-by-case application of multiple and combined strategies (*e.g.*, written educational material, nurse facilitation of the process, *etc.*) may have the potential to influence the outcome[45,48].

Elderly

With the extension of life expectancy, the opportunities for medical care for elderly patients increase [49]. The rise in cancer patients, in particular, is a global challenge; the International Agency for Research on Cancer estimates that the number of cancer patients will rise dramatically[49]. Regardless of the procedure's risks, bowel preparation can be problematic in terms of purgative effect and risk[50].

Patients must also go through a period of dietary restriction, fasting, and later fluid restriction, which can be difficult for people who have diabetes or chronic kidney disease (CKD)[50]. Furthermore, an elderly patient whose only access to the toilet is *via* stair-lift may be unable to prepare at home, necessitating in-patient admission for bowel preparation or alternative investigation to colonoscopy[50].

Despite the fact that little research has been conducted on bowel preparation in the elderly and very elderly population, a 2016 study discovered that diabetes, difficulty walking, or performing activities of daily living were associated with poor bowel preparation in patients over the age of 65[50,51]. Furthermore, poor bowel preparation is the leading cause of colonoscopy failure in patients aged 90 and up[50,52].

In five years, a British Patient Safety Agency alert reported one death and 218 patient safety incidents [53]. Although the vast majority of these incidents (93%) resulted in no or minor harm, 6% resulted in moderate harm, and one patient died[53].

Omitted medication (29%), incorrect drug (23%), and incorrect or unclear dose, strength, or frequency (11%) were all considered medicine errors. Side effects of bowel preparation include hypovolaemia, renal failure, and electrolyte disturbances[54]. An accurate bowel preparation prescription, as well as consideration of potential interactions and side effects, is especially important in older adults, who are more likely to have multiple comorbidities and polypharmacy, both of which may exacerbate these side effects. They may also have less physical reserve to deal with any unforeseen complications. In patients over the age of 65, serum albumin concentration before bowel preparation, for example, may predict hypovolaemia[55].

In elderly patients with pre-existing chronic renal failure, all oral laxatives should be used with caution, and patients undergoing dialysis or with advanced CKD should consult with the renal team. Sodium phosphate preparations should be avoided entirely in patients with renal failure[54]. Furthermore, any medications that, when combined with bowel preparation, may worsen renal failure, such as diuretics, angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists, should be avoided[54].

Cleaning success was achieved in 70.3% of patients aged > 65 years with comorbidities undergoing colonoscopy after a 1-L PEG-ASC, 2-L PEG/PEG-ASC, or 4-L-PEG-based preparation, according to a recent retrospective analysis of a prospective cohort. Notably, elderly patients had a higher rate of inadequate colonoscopy cleansing than non-elderly patients (7.0% *vs* 3.8%, $P = 0.012$)[56]. Split regimen, adequate cleansing at last colonoscopy, tolerability score, a low-fiber diet for at least 3 days, and colonoscopy within 5 h after the end of preparation (OR = 2.43, $P = 0.003$; OR = 2.29, $P = 0.02$; OR = 1.29, $P < 0.001$; OR = 2.45, $P = 0.001$; OR = 2.67, $P = 0.008$, respectively) were predictors of bowel cleansing in the elderly[56]. Also, 1-L PEG had a higher tolerability score than 2-L and 4-L PEG in elderly (7.7 *vs* 7.2 and 7.2, $P = 0.099$). Interestingly, when compared to the other preparations, the 1-L PEG-ASC preparation was associated with higher quality cleansing of the right colon (39.6% *vs* 17.0% *vs* 9.4%, respectively, $P < 0.001$) and may thus be the preferred option for the elderly.

Methods for achieving safe and adequate bowel preparations in the elderly should include clear instructions, reminder calls, and case management for potential confounding patient-related factors[57].

Comorbidities

Endoscopic techniques have become increasingly important in recent years, particularly in the treatment of colorectal flat lesions[58,59].

At the same time, the significance of accurate detection and assessment of such lesions in predicting malignancy has become clear. Indeed, proper bowel preparation is critical for colonoscopy because it allows for visualization of the entire colonic mucosa and improves the safety of therapeutic maneuvers [6,60].

In contrast, poor preparation lengthens the procedure, increases the risk of complications, and increases the likelihood of missing lesions[61].

The percentage of colonoscopies performed with inadequate bowel cleansing ranges between 5% and 35%[12,44,62-66].

Because a proper bowel cleansing regimen increases the likelihood of success, identifying risk factors for inadequate bowel cleansing is critical. Patient-related predictors of colonoscopy preparation failure include prior inadequate bowel cleansing, a history of constipation, increasing age (> 65 years), male gender, low health literacy (*e.g.*, cognitive skills), inpatient status, obesity, diabetes mellitus (DM), inflammatory bowel disease (IBD), unexplained chronic diarrhea, megacolon, cirrhosis, stroke,

dementia or Parkinson's disease, patients at increased risk for electrolyte abnormalities (*e.g.*, patients on diuretics), uncontrolled hypertension, severe congestive heart failure (New York Heart Association class III or IV), severe CKD (creatinine clearance < 30 mL/min/1.73 m), previous colorectal surgery, use of constipation-related medications (narcotics and tricyclic antidepressants), severe colonic stricture or obstructing tumor or perforation, dysphagia, gastroparesis, or gastric outlet obstruction, pregnancy or lactation[12,64,65,67-69].

Administration of the entire preparation the night before the colonoscopy, rather than split-dosing, and a later start time for the colonoscopy are procedure-related risk factors for inadequate bowel preparations[44]. The presence of one or more of these risk factors can influence bowel cleansing regimens and choices[70].

ESGE recommends the use of high-volume or low-volume PEG-based regimens, as well as non-PEG-based agents that have been clinically validated for routine bowel preparation. For elective colonoscopy, split-dose bowel preparation (with or without additional measures) should be used, as it has been linked to improved preparation quality[13].

In 2015, Dik *et al*[71] conducted a Dutch study that included only patients who who received a split-dose regimen. In total, 1331 colonoscopies were included in the study, with 12.9% having insufficient bowel preparation. Diabetes, chronic constipation, a history of abdominal or pelvic surgery, and recent hospitalization are all risk factors for poor bowel cleansing quality.

Gandhi *et al*[65] conducted a meta-analysis of independent risk factors in over 75,000 people receiving a split-dose bowel preparation. Constipation, diabetes, and medication use were identified as predictors of colonoscopy preparation failure despite the studies' heterogeneity. In a 2018 meta-analysis by Mahmood *et al*[12], age, male sex, inpatient status, DM, hypertension, cirrhosis, narcotic use, constipation, stroke and tricyclic antidepressants were associated with inadequate bowel cleansing (OR = -1.20, OR = 0.85, OR = 0.57, OR = 0.58, OR = 0.58, OR = 0.49, OR = 0.59, OR = 0.61, OR = 0.51, respectively). Furthermore, in Western countries, diabetes, cirrhosis, male sex, stroke history, and tricyclic antidepressant use were found to be stronger risk factors for inadequate bowel preparation than in Asian countries.

In a 2022 United States retrospective study of 1029 patients, Agrawal *et al*[66] discovered the following factors to be associated with colonoscopy cancellations: Graduate school education, Hispanic ethnicity, a hemoglobin level of 10 g/dL, and if the colonoscopy was done for other indications (OR = 1.93, $P = 0.04$; OR = 0.47, $P = 0.01$; OR = 1.41, $P = 0.05$; OR = 0.53, $P = 0.04$, respectively). Dementia (OR = 2.44, $P = 0.02$) and gastroparesis (OR = 3.97, $P = 0.01$) were factors associated with poor bowel preparation in a multivariate analysis.

Ultimately, in a 2016 United States study of 2401 colonoscopies, African Americans were 70% more likely to have suboptimal preparation (95%CI 1.2-2.4); DM, tricyclic antidepressant use, narcotic use, and Miralax-Gatorade prep *vs* 4-L PEG 3350 were all associated with suboptimal preparation quality in a multivariable analysis (OR = 2.3, 95%CI 1.6-3.2; OR = 2.5, 95%CI 1.3-4.9; OR = 1.7, 95%CI 1.2-2.5; OR = 0.6, 95%CI 0.4-0.9, respectively)[72].

Obesity: Obesity, when combined with other risk factors, is an independent predictor of poor bowel preparation during a colonoscopy in practice.

In a 2013 retrospective study of 2163 consecutive patients, mostly men, who had colonoscopies in Indiana, one of the independent risk factors for inadequate preparation was a body mass index (BMI) of ≥ 30 Kg/m^2 (OR = 1.46, 95%CI 1.21-1.75, $P < 0.0001$)[73].

Sharara *et al*[74] discovered that BMI was an independent risk factor for inadequate preparation in a 2016 Arabic study involving 541 patients. Obesity was associated with an OR of 5.3 (95%CI 1.4-19.8, $P = 0.01$) when compared to normal BMI. In a prospective study of 195 patients, obese patients had comparable rates of inadequate preparation to normal-weight individuals (OR = 0.7, 95%CI 1.10-3.96, $P = 0.68$). Patients who were underweight performed significantly worse than those with normal BMI (OR = 8.0, 95%CI 1.1-58.0, $P = 0.04$).

A high BMI had a significant difference in the effect of bowel cleansing between studies with mostly female patients (OR = 1.05) and studies with mostly male patients (OR = 1.30) ($P = 0.013$ for the difference), according to a 2018 systematic review and meta-analysis[65]. Inadequate bowel preparation was linked to diabetes (OR = 1.79) and hypertension (OR = 1.25), among other risk factors.

According to a recent study by Passi *et al*[75] in the United States, 49.4% of 27696 colonoscopies had insufficient bowel preparation, which was most common in the class III obesity group. When compared to the normal body mass index (BMI) group, a BMI of 30 kg/m^2 and 40 kg/m^2 was associated with an increased risk of an incomplete colonoscopy ($P = 0.001$ for overweight, $P = 0.0004$ for class I/II obesity), a longer procedure ($P < 0.05$ for all), and poorer tolerance ($P < 0.0001$ for class I/II obesity, $P = 0.016$ for class III obesity).

According to some studies, distinct bowel preparations are beneficial and safe for obese patients. In a 2012 prospective Australian study of 104 patients showing a similar bowel preparation quality after using sodium picosulphate, 90% of non-obese and 89% of obese patients had good bowel preparation ($P > 0.99$)[76].

Patients were randomized to receive split-dosing of either NER1006, 2-L PEG-ASC, or OSS in a recent (2021) two phases III Spanish trials[77]. Split-dose NER1006 (1-L-PEG-ASC) was associated with high

levels of cleansing, ranging from 87% to 94% in a total of 551 patients, including those who were obese or diabetic. Obese males aged above 60 had significantly higher overall and high-quality bowel-cleansing success rates with 1-L-PEG-ASC, at 100.0% and 72.7%, respectively, compared to 86.7% and 50.0% in the control group (P = 0.015 and P = 0.033, respectively).

Diabetes mellitus: Due to the high prevalence of gastrointestinal symptoms and the increased risk of CRC, diabetic patients have a higher demand for colonoscopies than the general population[78-80].

As a result, adults with diabetes should be properly screened, and a longer bowel preparation may be necessary to ensure an adequate endoscopic examination[81].

Due to dietary/medication regimen changes, narcotic use, and diabetes-related complications/comorbidities such as hypoglycemia, electrolyte imbalance, acute renal failure, and ketoacidosis, diabetic patients are at risk of poor bowel preparation[82,83].

DM has been identified as an independent risk factor influencing bowel preparation quality by decreasing colonic motility[71,84-86].

The rate of insufficient bowel preparation in diabetic patients ranges from 9% to 30%[84,87,88], which should be significantly reduced by implementing a multifactorial strategy. Surprisingly, even though DM patients are notoriously difficult to prepare, few studies have looked into the best bowel preparation management strategy in this setting. In diabetic patients, taking 10 ounces of magnesium citrate two days before colonoscopies, in addition to a single 4-L PEG dose, improved colon cleansing (from 54% to 70%)[89].

Another single-blind prospective trial on DM patients discovered that adding lubiprostone, a highly selective locally-acting activator of chloride channels used in functional constipation, to a single 4-L PEG the day before the procedure improved colon cleansing; however, the improvement was statistically non-significant due to the small sample size[90]. A small trial in DM outpatients examined additional bowel cleansing strategies with 6-L PEG, but the results were not encouraging[84].

Current United States guidelines do not endorse any of these recommendations, instead recommending a split-dose bowel cleansing regimen for DM patients with no adjustments[8]. A subsequent European randomized, single-blind, superiority trial compared a conventional bowel preparation protocol with a diabetic-specific preparation protocol, which included a low-fiber diet for three days, a clear liquid diet for one day, and a 4-L split-dose PEG regimen[88].

The latter group was given a special education program that included diet, laxative intake, and blood glucose-lowering agent adjustment instructions. In the conventional protocol, inadequate bowel cleansing was statistically more common than in the diabetic-specific protocol (20% *vs* 7%; RR = 3.1, 95%CI 1.2-8.0, P = 0.014).

Chronic constipation: The most prevalent type of constipation, functional chronic constipation, frequently affects women and the elderly who undergo colonoscopies often and ranges in prevalence from 2% to 27% in Western countries[91-93]. Constipation has been identified as a risk factor for inadequate bowel preparation[13,94]. Currently, ESGE does not recommend any specific bowel preparation in patients suffering from constipation chronically[13].

In elderly patients, slow transit constipation, defined by decreased bowel movements, may result in insufficient laxative wash-out and bowel preparation. This hypothesis was confirmed in a 2015 Korean study[95], which discovered that colonic transit time of more than 30 h was associated with inadequate bowel preparation. Furthermore, slow-transit constipation, as determined by radiopaque marker colonic transit testing, was linked to a more than 2-fold increased risk of poor bowel preparation in a 2022 study of 274 American patients with chronic constipation (OR = 2.2, 95%CI 1.1-4.4)[96].

In patients with a history of constipation, additional bowel purgatives should be considered[8]. Numerous studies in recent years have suggested different bowel preparation regimens in patients with chronic constipation, with good results using a variety of laxatives.

In a double-blind 2008 United States trial, 200 CRC screening patients were randomly assigned to receive a 24 g dose of lubiprostone or placebo before a split-dose PEG with electrolytes bowel preparation in the absence of dietary restriction[97].

Split-dose PEG, electrolytes, and lubiprostone pretreatment was found to be more effective (P = 0.001) and tolerable (P = 0.003) than placebo, most likely due to a reduction in abdominal bloating (P = 0.049)[97].

In a 2015 Italian randomized, single-blind study, 400 constipated patients were enrolled and randomly assigned to one of two arms: Split 2-L PEG-citrate-simethicone plus 2-day bisacodyl or split 4-L PEG[98]. In a 2016 Chinese RCT[99], the addition of lactulose one day before colonoscopy in combination with 4-L split-dose PEG was shown to be significantly superior (P < 0.05) to the conventional preparation with oral PEG and electrolytes for colonoscopy bowel preparation.

In terms of ease of administration (P < 0.001), willingness to repeat (P < 0.001), and compliance (P = 0.002), the 2-L PEG-citrate-simethicone/bisacodyl solution was found to be significantly more acceptable[98]. According to a 2019 RCT[100], the optimal dose of crystalline lactulose for Japanese constipated patients is 26 g/day. A short therapy cycle of PEG plus electrolytes was effective and safe in improving bowel preparation in chronic constipation patients in a 2020 and 2021 Japanese study[101,102]. In 2016, a larger Asian population was studied in a randomized, double-blind, placebo-controlled trial[103]. Surprisingly, when lower doses of PEG were combined with lubiprostone, no significant

difference in preparation quality was observed.

In a 2021 systematic review and meta-analysis of three RCTs, Dang *et al*[104] enrolled 225 chronically constipated patients, with 47.6% receiving sodium phosphate and 52.4% receiving PEG. Despite the low quality of evidence, patients who received sodium phosphate before their colonoscopy had cleaner colons than those who received PEG (OR = 1.87, 95%CI 1.06-3.32, P = 0.003).

IBD: Inadequate bowel preparation has also been linked to comorbidities such as IBD[105]. This was demonstrated in an Italian multicenter, randomized, single-blind study of 211 adult outpatients with ulcerative colitis (UC) undergoing colonoscopy and receiving either 2-L PEG plus bisacodyl or 4-L PEG [106]. Low-volume PEG was not inferior to 4-L PEG for bowel cleansing in UC (P = NS), but it was better tolerated (P < 0.0001) and accepted (P < 0.0001). The split dosage was associated with better cleansing regardless of preparation. A period of more than 6 h between the end of preparation and the colonoscopy predicted poor cleansing.

In a 2021 retrospective analysis of a prospective cohort, Maida *et al*[107] demonstrated the efficacy and safety of 1-L PEG-ASC in 45% of 411 patients.

IBD patients had higher cleansing success (92.9% *vs* 85.4%, P = 0.02) than controls, with a similar number of patients experiencing adverse events (22.2% *vs* 21.2%, P = 0.821) and treatment-emergent adverse events (51 *vs* 62%, P = 0.821). Furthermore, the presence of IBD (OR = 2.51, P = 0.019), lower age (OR = 0.98, P = 0.014), a split regimen (OR = 2.43, P = 0.033), the absence of diabetes (OR = 2.85, P = 0.015), and chronic constipation (OR = 3.35, P = 0.005) were all independently associated with cleansing success[107].

Endoscopic disease activity has recently been discovered to predict suboptimal bowel preparation, and biological therapy has been shown to protect IBD patients from it.

In a 2022 United States study by Kumar *et al*[108], the moderate-to-severe endoscopic disease was associated with higher odds of suboptimal bowel preparation *vs* mild or inactive disease [adjusted OR (aOR) 2.7; (95%CI 1.52-4.94)], whereas baseline biologic use was associated with a lower odds of suboptimal bowel preparation [aOR, 0.24 (0.09-0.65)] among the overall IBD cohort. Furthermore, age > 65 years and single-dose *vs* split-dose bowel preparation were independent predictors of suboptimal bowel preparation [aOR, 2.99 (1.19-7.54); aOR, 2.37 (1.43-3.95), respectively].

Liver cirrhosis: Liver cirrhosis predicts poor bowel preparation at screening colonoscopy[64,109].

This finding is most likely due to multiple factors impairing intestinal motility in cirrhotic patients [110,111]. The role of chronic liver disease in predisposing to inadequate bowel preparation in the absence of cirrhosis is unknown. In a 2016 United States study, Anam *et al*[112] compared 120 cirrhotics to 220 non-cirrhotics with chronic liver disease, and the first group performed significantly worse on bowel preparation. Cirrhotics had lower bowel preparation scores than non-cirrhotics (P = 0.0027), with cirrhotics having the lowest (48%) and non-cirrhotics having the highest (30%), with no effect of the MELD score.

The rate of failure to complete the bowel preparation and the incidence of side effects were comparable in 53 cirrhotics compared to 52 healthy subjects undergoing screening colonoscopy, according to an Italian 2015 study by Salso *et al*[113]. Despite this, nearly half of the cirrhotics (49% *vs* 5% control; P < 0.001) had poor bowel cleansing.

In a 2017 Chinese retrospective study, Lee *et al*[114] compared the safety of two bowel-cleansing agents in patients with liver cirrhosis (2-L PEG-ASC *vs* 4-L PEG). Patients preferred the 2-L PEG-ASC over the 4-L PEG group for acceptability and compliance. Finally, because both groups were successfully cleansed, the authors concluded that using 2-L PEG-ASC for colonoscopy in cirrhotics was a safe option.

Decompensated cirrhosis patients are more prone to frailty, cognitive abnormalities, and decreased ambulation. Clayton *et al*[115] discovered that patient educational video did not improve bowel preparations (split-prep) in the pre/post-intervention period in 121 patients with decompensated cirrhosis undergoing colonoscopy during the initial liver transplantation evaluation (29.8% *vs* 31.9%, respectively).

Furthermore, patients with moderate to severe ascites had a significantly higher rate of inadequate colonoscopy bowel preparation than non-ascites patients[115].

CKD: The use of cleansing agents in patients with CKD should be carefully evaluated due to the risk of electrolyte imbalance or worsening renal function[116]. No significant changes in vital or biochemical parameters have been linked to high volume osmotically balanced solutions containing PEG and electrolytes capable of maintaining bowel lumen isosmosis[13].

According to previous research[117,118], PEG is generally safe in CKD patients; however, adequate hydration and renal function monitoring should be ensured before and after colonoscopy in some cases to avoid acute kidney failure[119]. Individualized laxative choice is strongly advised for patients at risk of hydroelectrolyte disturbances (moderate quality evidence)[13].

Because of hyperosmolarity and the risk of magnesium toxicity, as well as acute phosphate nephropathy, magnesium-based preparations and sodium phosphate should be avoided in CKD patients[13,120,121].

Furthermore, due to the poor tolerability of high-volume PEG-based regimens, low-volume PEG (2-L) solutions with ascorbic acid (PEG-ASC) solutions have been proposed to reduce the patient's excessive fluid intake. Ascorbic acid can act as an osmotic agent and enhance the laxative effect of PEG due to its hexose structure[122], and its pleasant taste makes it easier for patients to swallow. Ascorbic acid, on the other hand, has been linked to the formation of renal stones and acidosis, with contradictory results[123, 124]. As a result, low-volume preparations continue to be a challenge for many CKD patients.

Notably, ESGE guidelines do not recommend aspartame and ascorbate-containing solutions (such as 2-L and 1-L PEG-ASC solutions) for patients with renal insufficiency and creatinine clearance less than 30 mL/min. A high rate of hypernatremia has been observed following the administration of 1-L PEG-ASC, owing primarily to the product's sodium content[13]. If low volume PEG solutions combined with citrate and simethicone are administered to patients with creatinine clearance less than 30 mL/min, caution is advised[13].

In a 2016 retrospective study, a same-day 1-L low-volume PEG regimen with a previous-day low-residue diet and laxative was tested to improve tolerability[125]. The study included 5,427 patients who were instructed to consume a low-residue fiber diet with 10 mL sodium picosulfate one day before the colonoscopy, followed by 1-L low-volume PEG and 0.5-L water four hours before the exam. In 86 CKD patients (creatinine 1.1 mg/dL), the BBPS 6 success rate was 94.1%, and there were no serious complications[125]. Lee *et al*[123] found that the 2-L PEG-ASC was a safe choice for bowel preparation before colonoscopy in patients with impaired renal function in a 2016 study.

In one retrospective cohort, patients with a GFR of 60 mL/min were given either 4-L PEG or 2-L PEG-ASC solutions. Patients in the 2-L PEG-ASC group (n = 61) rated their tolerance and acceptability higher than those in the 4-L PEG group (n = 80)[123]. After either preparation, there was no statistically significant change in electrolytes, blood urea nitrogen, or creatinine. When the regimens were compared, 7.5% of 4-L PEG patients and 11.5% of 2-L PEG-ASC patients had a transient > 30% increase in creatinine levels, though the differences were not statistically significant[123]. Ohmiya *et al*[126] discovered that same-day conventional bowel preparation with PEG electrolyte lavage solution plus Ascorbate (PEG-ELS-ASC) was safe and effective in 56 CKD patients in the Japanese 2021 study.

Only retrospective cohorts have found PEG to be safer than other formulations in patients with impaired renal function[127].

The most severe kidney injury case reported reversible post-colonoscopy acute renal failure within a few weeks of oral sodium phosphate (OSP) intake, necessitating renal replacement therapy in 19% of patients[128]. Furthermore, during the 2006-2007 time period, the Food and Drug Administration received reports of 171 cases of renal failure caused by the use of OSP and 10 cases caused by the use of PEG[128]. A 2005 retrospective population-based Iceland study found that the risk of biopsy-proven acute phosphate nephropathy is about one in every 1000 OSP doses sold[128].

Three RCTs comparing OSS preparation to 4-L PEG found that split OSS was noninferior to split high-volume PEG in terms of efficacy, safety, and tolerability. Although real-world data on OSS in the setting of renal insufficiency are limited, and despite no significant differences in the frequency of acute renal failure reported with this preparation, European guidelines[13] recommend that it be avoided in patients with severe renal insufficiency (glomerular filtration rate 30 mL/min).

According to ESGE guidelines and current evidence, patients with severe renal insufficiency should be prepared with isotonic high volume PEG solutions rather than low volume PEG or non-PEG regimens.

Heart disease: Previously, it was thought that bowel preparation (particularly after administration of PEG-ELS solution) could worsen heart failure[129], as a result, except in urgent or emergency cases, exposing such patients to a colonoscopy was risky. Also, coronary heart disease has been identified as a risk factor for severe desaturation and relevant electrocardiographic changes during endoscopic sedation[130]. Furthermore, several studies have found that these solutions may be harmful to patients with heart disease due to the potential increase in plasma volume and their effects on electrolyte disturbances[131].

Thiazide diuretics and SSRIs, which have the potential to cause fluid and electrolyte imbalances, should be avoided in at-risk patients while undergoing bowel preparation[132].

Heart disease and CRC were the only predictors strongly associated with poor bowel cleansing in a 2019 Spanish single-center, endoscopist-blinded RCT of 136 patients (OR = 3.37, 95%CI 1.34-8.46, P = 0.010; OR = 3.82, 95%CI 1.26-11.61, P = 0.018, respectively)[133]. In a 2020 study, Poola *et al*[134] discovered that 44% of 315 inpatients' bowel preparation was fair/poor. Poor bowel preparation was associated with elderly people who had a history of congestive heart failure.

Concomitant medications

Most medications can be taken with a small sip of water until the day of the colonoscopy. Because of the decreased oral intake prior to the procedure, some medications, such as diabetes medications or anticoagulants, may need to be adjusted. Because it causes residual feces, oral iron should be stopped at least five days before the colonoscopy[135]. Additionally, the procedure's urgency and the availability of alternative tests must be taken into account.

In a 2017 Chinese systematic review and meta-analysis of RCTs (overall 3217 patients with chronic pain), Huang *et al*[136] discovered that treatment with a fixed-ratio combination of prolonged-release oxycodone/naloxone reduces the incidence of opioid-induced constipation and provides clinically significant intermediate-term bowel function improvement while maintaining pain relief. According to a 2019 Spanish study by Velázquez Rivera *et al*[137], tapentadol and oxycodone had better bowel function profiles with no differences in a cross-sectional observational study of 180 Spanish patients with opioid-induced constipation during long-term treatment.

Previous history of colorectal surgery

Inadequate bowel cleansing is detrimental to the examination, resulting in lower ADR, longer procedural time, lower rates of cecal intubation, shorter intervals between examinations, and a 12%-22% increase in overall colonoscopy cost[4]. Previous colorectal surgery is a risk factor for patients who are difficult to prepare[64,138,139].

Previous colorectal surgery (along with diabetes, Parkinson's disease, and liver cirrhosis) is a condition that significantly predicts inadequate colon preparation, according to a prospective study enrolling 2811 outpatients in 2012[64].

Another study published in 2015 discovered that diabetes, chronic constipation, a history of abdominal and/or pelvic surgery, and current hospitalization were all significant predictors of poor bowel cleansing in patients using a split-dose preparation scheme[71]. Improved bowel preparation strategies may be difficult to achieve in this patient population. The long-term effects of colonic surgery and anastomoses on colonic motor function are currently unknown[140]. Patients who have had colonic surgery are usually excluded from trials on the efficacy of bowel preparations due to this lack of knowledge. As a result, no evidence-based guidelines for preparing this subset of patients are now available, and the various bowel cleansing schemes rely on single-center experience or expert opinions recommending high-volume regimens[141]. Following surgery, some mechanisms affecting the enteric nervous system and autonomic innervation are known to be altered.

Indeed, Vather *et al*[142] discovered that distal colonic motor patterns traversed healed anastomosis sites regularly, indicating possible cellular regeneration. Some of the causes could be attributed to colorectal anastomoses' effect on the enteric nervous system and autonomic innervation, which can result in changes in colonic retrograde and antegrade motor patterns[142-144]. Remarkably, patients who have had a previous colectomy appear to be the most difficult to prepare. While right colectomy reduces absorption and determines rapid transit, left colectomy may worsen peristalsis in moving luminal content outside the body[145].

Mussetto *et al*[139] found that a low-volume mixed preparation (15 mg bisacodyl plus 2-L PEG) was not inferior to 4-L PEG for adequate bowel cleansing during surveillance colonoscopy in a 2015 study of 120 patients who had prior colorectal resection for cancer (85.0% *vs* 81.7%, $P = 0.624$). Notably, the mixed low-volume regimen had a higher success rate and tolerability in patients who had previously undergone left colectomy *vs* right colectomy ($P = 0.025$ and $P < 0.001$, respectively). The only predictor of unsuccessful cleansing using logistic regression was previous left colectomy ($P = 0.012$). Similarly, Yoo *et al*[146] conducted a case-control study in Turkey in 2018, enrolling 200 patients who received either a low-volume or high-volume bowel preparation regimen. In terms of adequate cleansing (modified BBPS of 6-9), there was no statistical difference between the resection and control groups (88% *vs* 88%). Patients with a left colon resection had an OR of 0.27 ($P = 0.003$) for successful cleansing, according to the logistic regression analysis of the resection group, and low-volume preparation (OR = 3.09, $P = 0.023$) was the best predictor of an effective cleansing procedure.

According to Kim *et al*[147], 12.1% of the 12,881 participants in the National Cancer Center's health screening cohort who underwent screening or surveillance colonoscopy had a history of abdominopelvic surgery. Poor bowel preparation was linked to gastric or minor intestinal surgery in a multivariate analysis (OR = 1.76, 95%CI 1.23-2.53, $P = 0.002$). Other types of surgery, on the other hand, did not affect bowel preparation quality.

Chung *et al*[148] looked at 247 patients who had previously had a colorectal resection and had a surveillance colonoscopy in a 2017 study. The right colon preservation group had a significant association with bowel cleansing quality in both univariate (22.3% *vs* 7.5%, $P = 0.028$) and multivariate (OR = 3.6, 95%CI 1.0-12.3, $P = 0.038$) analysis.

In contrast, Gandhi *et al*[65] discovered that a history of abdominal surgery (OR = 0.99) did not correlate with inadequate bowel preparation in a 2018 systematic review and meta-analysis of 67 studies involving 75818 patients.

A study (NCT02761317) is currently being conducted on patients who have had colorectal resection to compare the efficacy of bowel cleansing between the standard preparation (2-L PEG solution, 2-L PEG-ELS), low-volume preparation (10 mg bisacodyl plus 2-L PEG-ELS), and high-volume preparation (10 mg bisacodyl plus 2-L PEG-ELS) (4-L PEG-ELS).

History of poor bowel preparation

The presence of one or more risk factors for inadequate preparation will influence preparation selection and regimen. Due to the high risk of missing clinically relevant lesions, patients with insufficient cleansing must have the colonoscopy repeated after a more thorough bowel cleansing attempt[13].

Patients who did not prepare adequately because they misinterpreted the instructions can be counseled and then directed to repeat the same bowel regimen. For patients who did not tolerate or respond adequately to the original preparation[149] alternative preparations should be tried.

Despite the lack of strong evidence from RCTs, the ESGE guidelines and some studies recommend repeating colonoscopy on the same or the next day with additional bowel cleansing (*e.g.*, 500 mL PEG-ASC) or using enema as a salvage option in patients with insufficient bowel cleansing[72,149-152]. The bowel preparation regimen outlined below should be tailored to the potential causes of failure[13].

Some patients continue to have inadequate preparation despite following the regimen, switching regimens, and using a split-dose lavage. In such cases, two days of clear liquids are usually prescribed, followed by a morning procedure. If the patient's preparation was extremely poor, options include adding a second laxative if contraindications exist (*e.g.*, using 4-L PEG-ELS solution followed by a 1-L solution of magnesium citrate) or repeating the preparation administration over two days (except for sodium phosphate).

Within one year, 90% of patients who did not do adequate bowel cleansing require a repeat colonoscopy after one or more attempts at bowel cleansing[13,70]. Similarly, experts advise patients who did not tolerate or respond well to the first bowel cleansing to try another. Split-dosing should also be used for improved bowel cleansing[70]. Despite adherence to the regimen, switching to different regimens, and using a split-dose lavage, some patients continue to have insufficient bowel cleansing for the next exam, particularly those with severe comorbidities (*e.g.*, stroke, dementia, DM, obesity), the elderly, men, and people taking psychotropic drugs, all of which are commonly associated with a chronic constipation condition[6,64,153]. The efficacy of a next-day or same-day colonoscopy after additional bowel preparation *vs* a later colonoscopy is limited and contradictory. In a 2009 Israelian single-center study of 235 patients with inadequate preparation, next-day colonoscopy was associated with a lower risk of secondary failure (OR = 0.31, 95%CI 0.1-0.92)[63]. In a 2013 retrospective study of 3047 procedures with inadequate cleansing, patients advised to have a next-day colonoscopy were more likely to follow the recommendation for a repeat colonoscopy[154]. In a larger 2016 United States single-center series of 397 patients with inadequate procedures, recurrent failure was observed in 30.0% of the next-day group and 23.5% of the non-next-day group (P = 0.48)[155].

In a single-center Korean prospective nonrandomized study conducted in 2014, 87 patients with insufficient preparation after an initial 4-L PEG were given either an additional 2-L PEG on the same day or a 4-L PEG plus bisacodyl one week later after 3 days of a low residue diet, with no difference found between the two regimens[149]. A 2017 Spanish randomized trial demonstrated the superiority of a high-volume PEG-based regimen over a low-volume PEG-based regimen when combined with an intensive preparation regimen[150]. In a 2018 observational study, 60 Turkish patients who had received inadequate preparation underwent a same-day repeat colonoscopy after receiving an additional laxative of 250 mL senna alkaloids with 1.5-L water, and 83% of patients had the repeat colonoscopy reach the cecum[151].

In a 2012 United States study, Sohn *et al*[152] found that direct administration of laxative enemas through the colonoscope into the right colon *via* the biopsy channel was effective in 21 patients with inadequate preparation after low-volume PEG and bisacodyl preparation. In a 2012 Japanese study, Horiuchi *et al*[156] published a study in which 26 patients with inadequate preparation after low-volume PEG were given 500 mL of PEG *via* the colonoscope biopsy channel, with 96.1% (25/26) of cases successfully prepared.

Yang *et al*[157] on the other hand, conducted a randomized trial in 125 patients with inadequate preparation, comparing administration of a 1-L PEG enema *via* colonoscope to additional oral ingestion of 2-L PEG, with 35 of 66 (53%) of the enema group and 53 of 67 (81%) of the oral group obtaining suitable preparation. 42 patients with inadequate preparation were randomly assigned to either pump irrigation or syringe irrigation in a 2012 Belgian monocenter study. In terms of pre-procedure preparation, pump irrigation outperformed hand irrigation, with a significant difference in the right colon[158].

Predictive models of inadequate bowel preparation in outpatients

Identifying risk factors for poor bowel cleansing can aid in determining which patients require more intensive bowel preparation. Six predictive models of inadequate bowel preparation have been developed to date based on patient potential risk factors[64,71,159-162].

Hassan *et al*[64] identified several factors that can significantly influence preparation quality in a 2012 Italian multicenter prospective study of 2811 consecutive outpatients, which were then used to develop an accurate predictive model. Overweight, male sex, having a high BMI, older age, previous colorectal surgery, cirrhosis, Parkinson's disease, diabetes, and positive fecal occult test results were all associated with inadequate bowel preparation. With 60% sensitivity, 59% specificity, 41% positive predictive value, and 76% negative predictive value, these factors predicted which patients would have inadequate cleansing.

In the validation cohort, the scale's discriminative ability was strong, with the area under the curve (AUC) of 0.77. An ASA score of 3, use of tricyclic antidepressants or narcotics, diabetes, constipation, previous abdominal and/or pelvic surgery, history of inadequate bowel preparation, and hospitalization were identified as independent predictors of inadequate bowel preparation in a 2015 Dutch

study by Dik *et al*[71]. The scale's discriminative ability was strong in the validation cohort, with an AUC of 0.77. Finally, in the multivariate analysis of 667 consecutive Spanish outpatients in 2017, antidepressants (OR = 4.25, 95%CI 1.91-9.47), co-morbidity (OR = 3.35, 95%CI 2.16-5.18), constipation (OR = 2.09, 95%CI 1.29-3.40), and abdominal/pelvic surgery (OR = 1.60, 95%CI 1.03-2.47) were independent predictors of inadequate cleansing. According to these findings, the model with all of these variables had an AUC of 0.72 in the development cohort and 0.70 in the validation cohort of 409 patients[150].

Berger *et al*[160] developed a predictive score called "Prepa-Co" in a recent French single-center study, allowing the identification of patients at high risk of inadequate bowel preparation. In total, 561 patients were included, with 25% having inadequate bowel preparation. In the prediction model of inadequate bowel preparation, the risk score includes seven variables: Diabetes or obesity, irregular physical activity, cirrhosis, use of antidepressants or neuroleptics, use of opiate medication, surgery history, and history of inadequate bowel preparation. With an AUC of 0.62, Prepa-Co correctly predicted bowel cleanliness in 68.3% of cases, with a specificity of 75.8% and a negative predictive value of 80.8%.

Sadeghi *et al*[161] conducted a population-based study on 2476 Iranian adults in 2022, and age, gender, ethnicity, BMI, abdominal circumference, fruit consumption, smoking, NSAIDs, SSRIs, education, constipation, physical activity, and diabetes were all factored into the predictive model, with the AUC reaching 0.70 in the final step.

Kurlander *et al*[162] developed prediction models for bowel preparation inadequacy in a retrospective cohort of 6885 United States veterans who underwent colonoscopy. The AUC for the validation cohort was 0.66 (95%CI 0.62-0.69), whereas the AUC for the validation cohort using random forest machine learning was 0.61 (95%CI 0.58-0.65).

So far, none of these predictive models have been tested outside of their validation cohorts, and no study has attempted to use a different regimen on patients who have risk factors for poor colon cleanliness. Furthermore, the ESGE concluded in 2019 that there was insufficient data to recommend the use of specific predictive models for inadequate bowel preparation in clinical practice[13].

CONCLUSION

To date, numerous efforts have been applied to increase the sensitivity of colonoscopy in the detection of polyps and advanced adenomas, reducing the risk of I-CRC[163,164]. These include the application of key-performance quality measures for colonoscopy[165], the use of distal attachment devices[166,167], and new intestinal preparations improving the quality of bowel cleansing[168].

Despite these efforts, adequate bowel cleansing remains a basic prerequisite for a quality colonoscopy, since it can affect other quality measures including ADR and cecal intubation rate.

Prior inadequate bowel cleansing, chronic constipation, elderly, male gender, low health literacy, obesity, diabetes, IBD, cirrhosis, neurologic disease, risk of electrolyte abnormalities, severe heart and renal failure, previous colorectal surgery, use of constipation-related medications, gastroparesis, and severe colonic stricture are the major patient-related predictors of colonoscopy preparation failure.

In the elderly, a strategy including a prolonged low-fiber diet, split preparation regimen, and colonoscopy within 5 h of the end of preparation, may increase the cleansing success rates. Furthermore, even though no specific product is specifically recommended in the other cases for difficult-to-prepare patients, recent evidence suggests that 1-L PEG-ASC preparation may be preferred in hospitalized and IBD patients. Figure 2 depicts a schematic view of the main bowel preparation tips to achieve a successful bowel cleansing.

According to current guidelines, patients with severe renal insufficiency (creatinine clearance less than 30mL/min) should be prepared with isotonic high volume PEG solutions, whereas low volume PEG plus adjuvants (*e.g.*, 1-L/2-L-PEG-ASC, and 1-L PEG plus citrate) or non-PEG regimens (*e.g.*, MCSP or OSS) are not advised.

To determine the most effective bowel preparation for difficult-to-prepare elderly, constipated, and cirrhotic patients, more high-quality research, including prospective studies with randomized designs, is required. Larger, multicenter, prospective studies are needed to determine the best bowel preparation for patients with prior abdominal-pelvic surgery, most importantly to improve ADR, especially for flat lesions in the right colon in patients who had a left colon resection.

Moreover, regardless of type of bowel solution, in very elderly patients with comorbidities, a careful assistance from family members or those who care for the elderly is necessary to guarantee the compliance to diet and preparation modalities.

In conclusion, an accurate characterization of procedural and patient variables may lead to a more personalized approach to bowel preparation (Table 1), reducing the risk of missed lesions and of I-CRC.

Table 1 Recommendation for bowel preparation in specific clinical settings

Clinical scenario	Remarks
Hospitalization	PEG-based regimens should be considered first in any preparation strategy because they are more likely to achieve adequate bowel cleansing while maintaining an optimal patient safety profile[13,45,48]
	Furthermore, multiple, combined strategies (*e.g.*, written educational material, nurse facilitation of the process, *etc.*) based on a case-by-case decision could influence the outcome[45,48]
	Although no specific product is strongly recommended for difficult-to-prepare patients, clinical evidence suggests that a 1-L PEG-ASC preparation may be preferred in hospitalized patients[22]
Elderly	A strategy that includes a low-fiber diet for an extended period of time, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may improve cleansing success rates in the elderly[57]
	A 1-L PEG preparation may be preferred for the elderly due to higher cleansing quality and higher compliance due to lower volume[56,57,77]
Obesity	ESGE recommends the use of high volume or low volume PEG-based regimens, as well as non-PEG-based agents that have been clinically validated for routine bowel preparation[13]
	For elective colonoscopy, split-dose bowel preparation (with or without the additional measures) should be used, as it has been linked to improved preparation quality[13]
Diabetes mellitus	Current US guidelines do not support assumption of lubiprostone or magnesium citrate, instead recommending a split-dose bowel cleansing regimen with no adjustments for DM patients[8]
Chronic constipation	ESGE does not recommend any specific bowel preparation in constipation patients[13]
Inflammatory bowel disease	Split dosage was associated with better cleansing regardless of preparation in some studies[106,108]
	1-L PEG-ASC is associate to higher cleansing success and good safety and should be preferred[107]
Liver cirrhosis	The use of 2-L PEG-ASC for colonoscopy in liver cirrhosis to be a safe option[114]
Chronic kidney disease	All oral laxatives should be used with caution in patients with pre-existing chronic renal failure and liaison with the renal team is advised in patients undergoing dialysis or with advanced chronic kidney disease[54,106]
	Individualized laxative selection is strongly recommended for patients at risk for hydroelectrolyte disturbances (moderate quality evidence)[13]
	Because of the risk of magnesium toxicity and acute phosphate nephropathy, magnesium-based preparations and sodium phosphate should be avoided in chronic kidney disease patients[54,120,121]
	Also, because high-volume PEG-based regimens are poorly tolerated, low-volume PEG (2-L) solutions with ascorbic acid (PEG-ASC) have been proposed to reduce the patient's excessive fluid intake[122]
	According to ESGE guidelines, patients with severe renal insufficiency (creatinine clearance less than 30 mL/min) should be prepared with isotonic high volume PEG solutions, whereas low volume PEG plus adjuvants (*e.g.*, 1L-, 2L-PEG-ASC, 1 L PEG plus citrate) or non-PEG regimens (*e.g.*, MCSP or oral sulfate solution) are not advised[13]
Heart disease	Bowel preparation (particularly after administration of PEG-ELS solution) could worsen heart failure[129]
Polypharmacy	Most medications can be taken up until the day of the colonoscopy and are taken with a small sip of water[135]
	Some medications, such as diabetes medications or anticoagulants, may need to be adjusted due to decreased oral intake prior to the procedure[13]
	Oral iron should also be discontinued at least five days before the colonoscopy because it causes residual feces[135]
History of colorectal surgery	In a study of 120 patients with prior colorectal resection for colorectal cancer, a low-volume mixed preparation (15 mg bisacodyl plus 2-L PEG) was not inferior to a high-volume regimen (4-L PEG) for adequate bowel cleansing during surveillance colonoscopy [139]
	In patients who had previously undergone left colectomy vs right colectomy, the mixed low-volume regimen had a higher success rate and tolerability[139,146]
History of poor bowel preparation	Despite a lack of strong evidence from randomized controlled trials, the ESGE guidelines and some studies recommend repeating colonoscopy using same-day or the next day with additional bowel cleansing (*e.g.*, 500 mL PEG plus ascorbate) or using enema as a salvage option in patients with inadequate bowel cleansing[73,149-152,156]
	The next bowel preparation regimen should be tailored to the potential causes of failure[13]

PEG-ASC: Polyethylene glycol plus ascorbate; MCSP: Magnesium citrate plus picosulphate; DM: Diabetes mellitus; ESGE: European Society of Gastrointestinal Endoscopy.

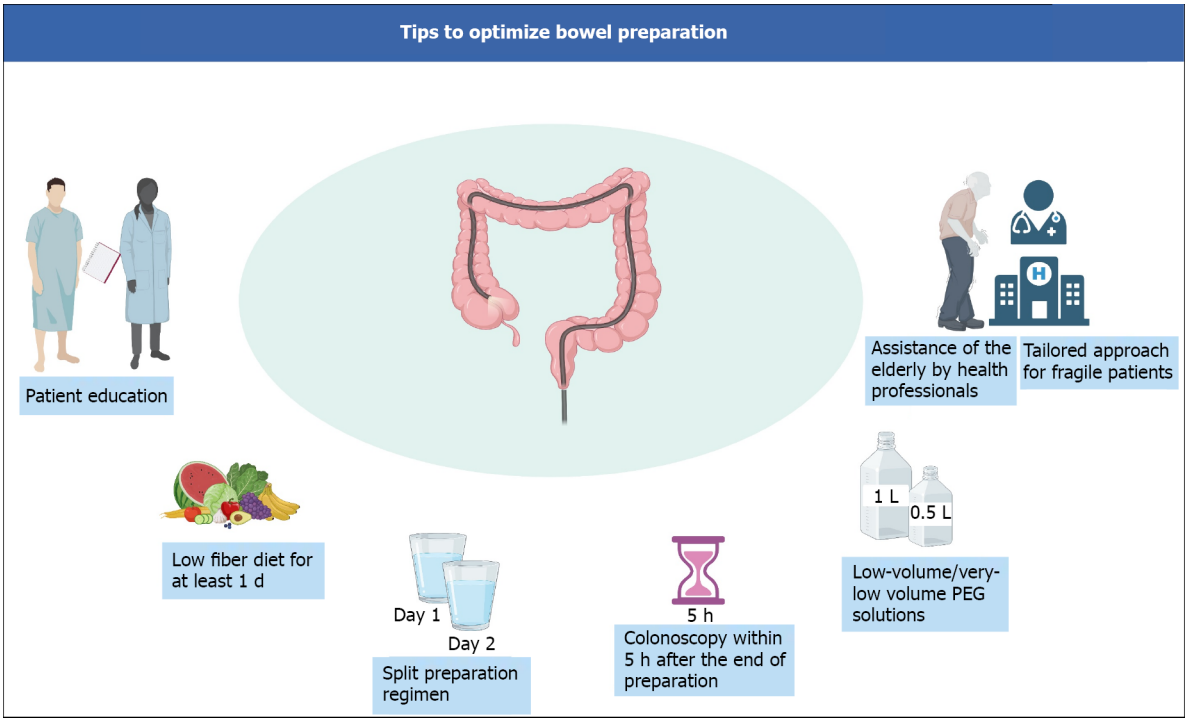


Figure 2 A schematic view of the main bowel preparation tips to achieve a successful bowel cleansing. PEG: Polyethylene glycol. PEG: Polyethylene glycol. Citation: The authors has been granted a license to use the BioRender content, including icons, templates and other original artwork, appearing in the attached completed graphic pursuant to BioRender's Academic License Terms, created with BioRender.com (Supplementary material).

FOOTNOTES

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MINIREVIEWS

Gut microbiome therapeutic modulation to alleviate drug-induced hepatic damage in COVID-19 patients

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Abstract

Coronavirus disease 2019 (COVID-19) infection caused by the severe acute respiratory syndrome coronavirus 2 virus, its symptoms, treatment, and post-COVID-19 effects have been a major focus of research since 2020. In addition to respiratory symptoms, different clinical variants of the virus have been associated with dynamic symptoms and multiorgan diseases, including liver abnormalities. The release of cytokines by the activation of innate immune cells during viral infection and the high doses of drugs used for COVID-19 treatment are considered major drivers of liver injury in COVID-19 patients. The degree of hepatic inflammation in patients suffering from chronic liver disease and having COVID-19 could be severe and can be estimated through different liver chemistry abnormality markers. Gut microbiota influences liver chemistry through its metabolites. Gut dysbiosis during COVID-19 treatment can promote liver inflammation. Here, we highlighted the bidirectional association of liver physiology and gut microbiota (gut-liver axis) and its potential to manipulate drug-induced chemical abnormalities in the livers of COVID-19 patients.

Key Words: COVID-19; Gut-liver axis; Probiotics; Prebiotics; Cytokines; Gut microbiome

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Core Tip: There are several reviews in the literature focused on the pathophysiology of liver damage during severe acute respiratory syndrome coronavirus 2 infection. However, we highlighted the potential role of gut microbiota in managing drug-induced liver damage during and after coronavirus disease 2019. We shed light on various metabolites produced by gut microorganisms that have a significant role in reducing liver damage in coronavirus disease 2019 with the use of different probiotics and prebiotics.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious viral disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that spreads easily from person to person *via* respiratory droplets in the form of aerosols. COVID-19 has a global overall mortality rate of 2%-3%[1]. After the first case was reported in Wuhan, China in November 2019[2], SARS-CoV-2 rapidly spread throughout the world. Because of the consequential health crisis worldwide, the World Health Organization declared COVID-19 a global pandemic disease in March 2020[3]. To date, more than 6 million deaths caused by the virus have been reported around the globe.

Similar to all other RNA viruses, when SARS-CoV-2 enters and adapts to a new human host, its nucleic acids mutate, which results in new viral progeny. Several variants have gained concern during the course of the pandemic due to their impact on human health, such as alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), and omicron (B.1.1.529)[4]. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) receptors of the host prior to entry into the cells. ACE2 is a protein receptor found on the epithelial lining of many cells and tissues such as the nose, mouth, and lungs. It is also present on blood vessels, the heart, kidneys, the liver, and the gastrointestinal (GI) tract[5,6].

Patients during the first outbreak of COVID-19 exhibited only respiratory symptoms. Approximately 2.6% suffered from diarrhea, and 2% suffered from chronic liver illness[7]. With the progression of the disease, several patients started to report gastric issues, including diarrhea, nausea, anorexia, vomiting, abdominal pain, anxiety, and intestinal bleeding[8]. These gastric symptoms lead to gut dysbiosis, which is associated with bacterial translocation into the blood during the course of COVID-19 infection, resulting in lethal secondary infections[9]. Similarly, COVID-19 damages the liver to various degrees. Numerous factors that could contribute to liver damage during infection include direct viral cytopathic effects, immune-mediated injury (systemic inflammatory response syndrome), passive hepatic congestion due to right-sided heart failure, liver hypoxia, and drug-induced liver injury[10]. Hence, COVID-19 is not limited to the respiratory tract but is also a multiorgan disease with dynamic symptoms.

To date, there is no effective therapy or antivirals for SARS-CoV-2 due to rapid genomic changes in the virus; however, symptoms are treated with various drugs. Clinical trials are being conducted to evaluate these drugs, although some of them have adverse effects on human health including liver damage or abnormal liver function[10]. It is well known that the use of drugs can cause dysregulation of gut microbiota (gut dysbiosis) as well[11]. Gut dysbiosis can result in hepatic inflammation through the biliary tract, portal vein, and systemic circulation. The translocation of endotoxins and bacteria due to increased intestinal permeability and reduction in the production of commensal gut microbial metabolites such as butyric acid, bile acids, phenolic compounds, indole and bile acid derivatives, and carotenoids promote liver inflammation. The intestine and the liver communication with each other through the gut microbiota and their metabolites have been highlighted in several studies[12]. In addition, the molding of gut community structure with drugs used for the treatment of COVID-19 infection is also well-documented[13,14]. It ultimately disturbs the gut-liver link and participates in the severity of COVID-19 consequently.

This review was conducted with the aim of evaluating and explaining the bidirectional association of the gut microbiota and liver and resolving drug-induced liver damage by investigating the role of the microbiota in restoring liver chemistry. We understand that the incorporation of microbiome-targeted therapeutics may potentially create a new way of alleviating or preventing drug-induced hepatic damage in COVID-19 patients.

SARS-COV-2 PATHOGENESIS

SARS-CoV-2 invades host cells by binding its spike protein to ACE2 host cell receptors, which is a transmembrane protein. More specifically, spike S1 of SARS-CoV-2 binds to the enzymatic domain of mACE2 (membrane-bound ACE2) of epithelial cells, resulting in the invasion of the virus in the form of endosomes. Since these membrane receptors are known to be expressed on the epithelial lining of the nose, lungs, GI tract, heart, liver, and blood vessels, they are prone to viral attachment[6]. Viral entry occurs *via* host proteases such as transmembrane serine protease types 2 and 4 followed by viral replication[15].

The SARS-CoV-2 virus also disrupts the normal intestinal microbiome, leading to digestive issues such as diarrhea. Meta-analysis of 60 studies comprising 4243 patients from China, Singapore, South Korea, the United Kingdom, and the United States showed a frequency of GI symptoms (nausea, vomiting, diarrhea, anorexia) of 17.6%. Among these, anorexia and diarrhea were most common at 26.8% and 12.5%, respectively. These symptoms usually appear after 1-2 d of respiratory symptoms (dry cough, sore throat, shortness of breath, pneumonia, and lung infection)[16,17]. Furthermore, there is an increase in the number of lung-derived C-C chemokine receptor 9 and cluster of differentiation 4 (CD4+) T cells in the small intestine by elevated levels of chemokine ligand 25, causing intestinal inflammation [18].

Several studies have shown the association of the GI tract in the pathogenesis of COVID-19, signifying the link between gut microbiota and the clinical outcome of the disease. Consequently, gut microbial dysbiosis has been found to be related to the development and severity of the disease[19,20]. When the gut microbial composition of patients with COVID-19 was compared with healthy individuals, numerous gut commensals with well-known immunomodulatory potential including *Faecalibacterium praunitzii, Eubacterium rectale,* and bifidobacteria were observed to be depleted in COVID-19 individuals and persisted to be low in stool and blood samples collected up to 1 mo after recovery from the disease. This unbalanced microbial composition indicated stratification according to disease severity corresponding to higher levels of inflammatory cytokines and blood markers including C-reactive protein, lactate dehydrogenase, γ-glutamyl transferase, and aspartate aminotransferase. The study not only specified the association of gut microbiome with disease severity but also indicated that altered gut microbiota may contribute to persistence of symptoms[20].

Similarly, Zuo *et al*[21] observed that the gut microbiome profiles of COVID-19 patients had prominent alterations with an increase in opportunistic pathogens such as *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* and a decrease in *Faecalibacterium prausnitzii* (butyrate producing anti-inflammatory bacterium), leading to severity of the disease[21]. Correspondingly, patients had higher proportions of opportunistic fungal pathogens in their fecal samples including *Candida albicans, Candida auris*, and *Aspergillus flavus* when compared to controls. Prolonged gut dysbiosis was observed during the hospitalization of these patients even after nasopharyngeal clearance of SARS-CoV-2, which indicates the long-term influence of the disease on the microbiota composition[21].

A cross-sectional study involving 30 patients suffering from COVID-19, 24 influenza A (H1N1) patients and 30 healthy individuals was conducted by Gu *et al*[22]. 16S rRNA analysis of V_3-V_4 regions revealed a decrease in gut microbial diversity of COVID-19 patients and a relative increase in opportunistic pathogens including *Streptococcus, Rothia, Actinomyces, Vellionella,* and *Erysipelatoclostridium* compared to healthy controls. Total number of *Streptococcus* and *Escherichia/Shigella* significantly increased in COVID-19 and H1N1 patients, respectively[22]. Disturbance in the normal gut microbiome and abundance of opportunistic pathogens leads to intestinal inflammation. Hence, the GI epithelium may become susceptible to SARS-CoV2 infection under certain circumstances such as viral load, coexisting disease, age, medication, gut dysbiosis, and inflammation[23]. Correspondingly, levels of interleukin (IL-17A) rise, triggering neutrophils to migrate. Hence, the lungs become prone to cytokines and bacterial invasions *via* the bloodstream, resulting in inflamed lungs[24].

The bidirectional axis of the intestine, microbiome, and liver *via* the portal vein is also affected. The portal vein transports host and microbial metabolites such as ammonia to the liver, which has an impact on liver functioning. Gut microflora that are involved in the fermentation of amino acids, constantly produce ammonia as metabolic waste, which is transported to the liver and converted into urea to be excreted in the urine. Opportunistic pathogens such as *Clostridium* and *Peptostreptococcus* are known to produce high levels of ammonia causing disruption in body nitrogen homeostasis. This eventually leads to hepatocellular metabolic dysfunction and liver injury[25] that might increase by gut dysbiosis during antiviral drug treatment of COVID-19 infection.

PATHOPHYSIOLOGY OF THE LIVER IN COVID-19 INFECTION

Despite the fact that SARS-CoV-2 is a respiratory infection, it is also primarily associated with the liver. As mentioned above, the presence of ACE2 receptors makes the host's liver prone to injury. The expression of the ACE2 receptor is significantly higher in cholangiocytes, *i.e.* 57.7% (bile duct epithelial cells) *vs* 2.6% in hepatocytes[26]. Multiple factors are involved in liver damage during COVID-19, such

as direct cytopathic attack by SARS-CoV-2, inflammation, intrahepatic immunity, multidrug-induced liver damage, drug toxicity, hypoxia, and gut dysbiosis (Figure 1)[27-30]. A direct viral attack can also lyse or induce hepatic apoptosis. Virus-specific protein 7a induces a caspase-dependent apoptosis pathway that is usually present in the lungs, kidneys, and liver[31]. Viral replication has also been observed in hepatic cells with viral spikes present in the host cytoplasm[32]. These observations suggest the cytopathic effect of SARS-CoV-2 on hepatocytes. Wang *et al*[33] further observed dilatation of the endoplasmic reticulum, reduced glycogen granules, mitochondrial swelling, and membrane disruption in hepatocytes followed by hepatic apoptosis and binuclear hepatocytes.

Another significant cause of liver damage is immune-mediated liver injury. It typically occurs due to a cytokine storm with elevated levels of IL-1, IL-6, viral-induced cytotoxic T cells (CD8), and tumor necrosis factor produced by host cells against viral infection[32,34-36]. Liver tissues of 40 patients who died due to COVID-19 were subjected to PCR for viral RNA in a study by Lagana *et al*[37]. Mitochondrial enlargement, dilation of the endoplasmic reticulum, and membrane dysfunction were observed in autopsies. Additionally, hepatic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found to be elevated to 68 U/L (normal 46 U/L) and 102 U/L (normal 37 U/L), respectively. Seventy-five percent of patients had macrovesicular steatosis, 50% had lobular necroinflammation (acute hepatitis), and 15% had sinusoidal microthrombi, indicating that COVID-19-infected individuals had biochemical evidence of liver damage.

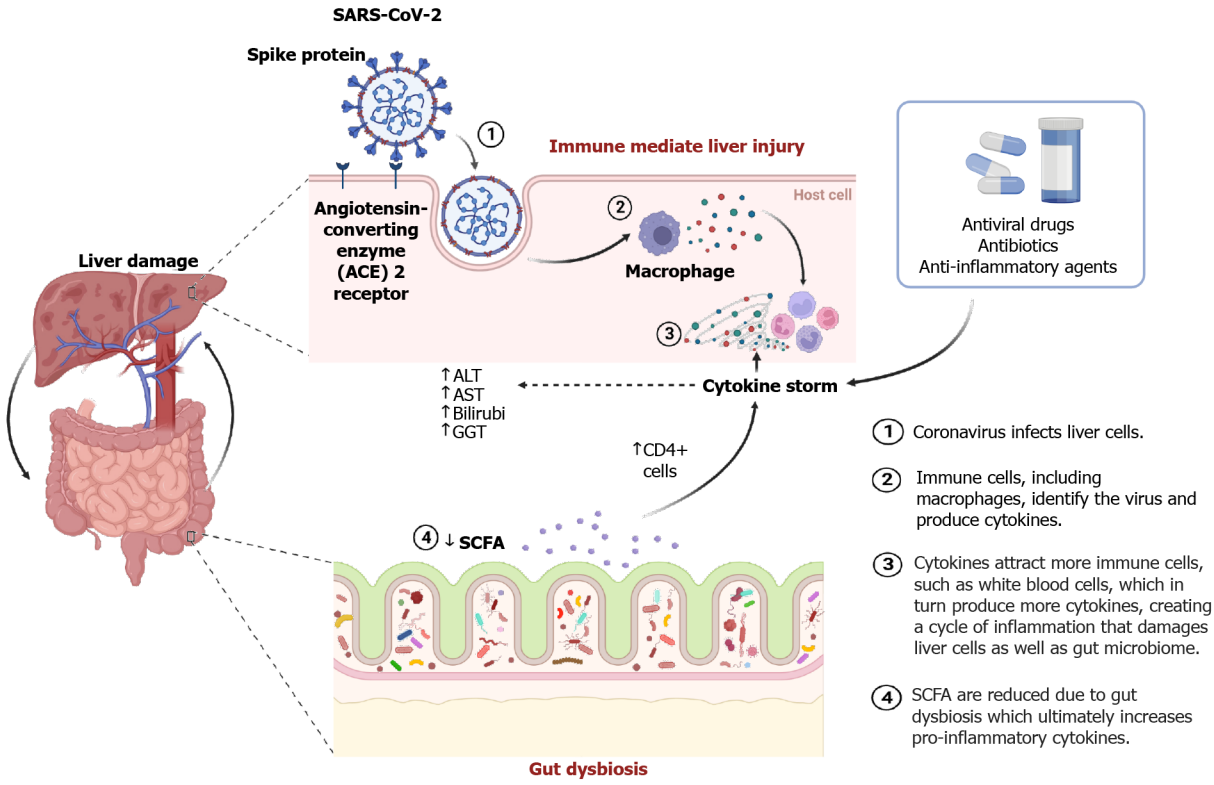
Neutrophils, Kupffer cells, and plasmocytes were observed in hepatic lobules and sinusoidal and portal regions in autopsies of 48 COVID-infected patients[38]. Liver damage related to COVID-19 infection results in a change in liver enzyme serum levels, with an increase ranging from 16% to 62% for aminotransferases and 5%-20% for bilirubin[39]. Another study by Guan *et al*[40] indicated increased aminotransferase levels in serum by 22% in 757 hospitalized patients, increased AST in 18.2% of patients with mild symptoms, 39% of patients with severe symptoms, and 50% of patients admitted to the intensive care unit (ICU). Serum bilirubin levels were also above the upper limit of normal in 13.3% of patients with mild symptoms and 20.8% of patients with severe symptoms[40].

DRUG-INDUCED LIVER DAMAGE (HEPATOTOXICITY)

The use of drugs for underlying chronic diseases, including antibiotics, antivirals, anti-inflammatory drugs, and anticoagulants, during COVID-19 infection is a major cause of liver damage (Figure 1)[41]. Cai *et al*[42] revealed that over 10% of the patients had elevated liver enzymes when they were admitted to the hospital, which may have been caused by prescribed medications[42]. A systematic review/meta-analysis consisting of 20874 SARS-CoV-2 patients summarized from 107 articles showed that 25.4% of patients had drug-induced liver toxicity. Among 208 patients who received remdesivir treatment, 15.2% had a drug-induced liver injury. Lopinavir/ritonavir had a higher incidence rate of 37.2% in 775 patients[43]. Furthermore, antiviral drugs (such as favipiravir, remdesivir, lopinavir/ritonavir, chloroquine, oseltamivir, and ribavirin) and antipyretics (acetaminophen) can lead to hepatotoxicity during the course of COVID-19 infection[34]. One of the most common causes of liver damage was underlying liver disease (chronic liver disease, hepatitis, cirrhosis, and non-alcoholic steatohepatitis), which was found in a meta-analysis of 13 studies including 3046 COVID-19 patients. Of these, 25% of individuals had a hepatic injury, 21% had elevated ALT, and 24% had elevated AST. These injuries lead to the severity of COVID-19 symptoms[44]. Therefore, it was suggested that patients with underlying liver diseases must not be prescribed hepatotoxic drugs since most of the drugs are metabolized in the liver, including oseltamivir, lopinavir/ritonavir, and chloroquines. Moreover, gamma-glutamyl transferase (GGT) and bilirubin levels mainly increase with the use of antiviral drugs in COVID-19 patients. The GGT enzyme is primarily found in liver cells. In the case of liver damage, the enzyme may leak out into the bloodstream, resulting in high levels of GGT in the blood and causing liver damage.

Drugs such as lopinavir (ritonavir) may cause a transient and slight increase in liver enzymes. Patients with advanced liver disease had elevated lopinavir plasma levels. Approximately 57.8% of patients taking lopinavir developed liver damage[45]. Lopinavir is a protease inhibitor. It is usually given in combination with ritonavir to increase the plasma half-life for the treatment of human immunodeficiency virus (HIV). Since it has low efficacy toward SARS-CoV-1, it must be prescribed as early as possible after the initial diagnosis of COVID-19[46]. Severe hepatotoxicity could be caused by a high dose of ritonavir (*i.e.* 1200 mg/day). However, in lower doses (200-400 mg), it could boost other drugs (such as lopinavir and indinavir)[47].

Lopinavir/ritonavir showed 63% adverse drug reactions in 217 COVID-19 patients. However, other drugs (umifenovir, chloroquine, and antibacterial) contributed to 47% of adverse drug reactions in total [48]. Fan *et al*[49] further indicated that out of 148 COVID-19 patients, 45 individuals had a normal baseline liver function test, among which 48% developed liver abnormalities after hospital admission. When compared to patients with normal liver function (31.3%), a significantly higher percentage of patients with abnormal liver function (57.8%) had received lopinavir/ritonavir after admission[49]. Similarly, in a study involving 417 COVID-19 patients, Cai *et al*[42] indicated that liver dysfunction was considerably more prevalent in the lopinavir/ritonavir-treated groups. Within 2 wk of admission, the



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Figure 1 Potential causes of liver injury during coronavirus disease 2019. Following severe acute respiratory syndrome coronavirus 2 infection, liver injury may arise due to direct viral entry [*via* angiotensin-converting enzyme 2 (ACE2) receptors on hepatocytes] or gut microbial dysbiosis leading to cytokine storm in the liver. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CD4+: Cluster of differentiation; GGT: Gamma-glutamyl transpeptidase; SCFA: Short-chain fatty acids.

presence of abnormal liver tests became more pronounced, with levels of ALT, AST, total bilirubin, and GGT exceeding the upper limit of normal in 49 (23.4%), 31 (14.8%), 24 (11.5%), and 51 (24.4%) patients, respectively. However, the definition of drug-induced liver injury by clinical guidelines from the European Association for the Study of the Liver was not applicable to this study due to the lack of evidence demonstrating the role of drugs in observed liver injury[42].

Simultaneous use of lopinavir/ritonavir and arbidol in COVID-19 patients with mild symptoms increased the odds of liver function, up to 3.58 times greater than in those who did not receive the medications. Human liver microsomes were used to examine the metabolic interactions between the two drugs in an effort to determine the cause of this unexpected increase. The following chain of evidence revealed that the use of tocilizumab was observed to improve both lung and liver functions within 3 wk in a case series of 7 patients who had significant abnormal liver tests in addition to worsening respiratory system function 5-7 d after receiving treatment with lopinavir/ritonavir, hydroxychloroquine, and azithromycin[50].

Since the direct effect of lopinavir/ritonavir on gut microbiota in SARS-CoV2 patients is still unknown, changes in bacterial diversity of HIV-1 patients have been observed when lopinavir/ritonavir was administered in antiretroviral therapy. Predominantly affected phylum include *Firmicutes*, *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria*. Moreover, levels of gut microbial genera such as *Lachnospira, Butyricicoccus, Oscillospir,* and *Prevotella* were reduced. The *Provetella* population has been previously linked to HIV-induced inflammatory response in the host[51]. Also, a decrease in *Lachnospira, Butyricicoccus,* and *Oscillospir* has adverse effects on the host immune system since these are beneficial microbiota of the human gut[52]. Short-chain fatty acids (SCFA) such as butyrate produced by gut microbiota suppress colon inflammation. Consequently, it protects against liver damage and regulates insulin signaling in adipose tissues[53,54]. Hence, the change in gut microbiota related to lopinavir/ritonavir treatment could pose a risk of liver damage during COVID-19 infection.

Another drug used for COVID-19 patients is remdesivir, which is a nucleotide prodrug of an adenosine analog. It terminates viral replication by binding to viral RNA-dependent RNA polymerase enzyme without interfering with host RNA or DNA polymerases[55]. Remdesivir has been used for the treatment of the Filoviridae viral family, including the Ebola and Marburg viruses[56]. Its efficacy extends to Lassa fever virus and pathogenic CoV (including Middle East respiratory syndrome and SARS CoVs)[2]. Grein *et al*[57] reported the first study in a cohort of 53 COVID-19 patients. Drug effects were observed from 5-10 d of administration. Elevated levels of hepatic enzymes with a 23% incidence

rate were the most frequent adverse effect. Additionally, elevated liver aminotransferase was the reason why 1 of the 4 patients stopped receiving treatment[57].

A similar pattern was observed in the study by Kalil *et al*[58] that included 402 patients to determine the best time course for intravenous remdesivir. The most frequent adverse effects on the liver in that grade were reported to be ALT and AST elevations of 1-2 (*i.e.* 7% and 6%, respectively). Moreover, Wang *et al*[33] demonstrated a placebo-controlled double-blind clinical trial on a group of 255 patients. Grade 1-2 elevated AST was identified as an adverse liver effect (12% in the placebo group, 7% in the drug-treated group), and grade 1-2 elevated ALT resulted in discontinuation of the drug (1%). Nevertheless, grade 1–2 hypoalbuminemia (15% in the placebo group, 13% in the drug-treated group) and grade 1–2 increased bilirubin (9% in the placebo group, 10% in the drug-treated group) were the most frequent liver adverse effects[33].

Interestingly, the harmful effects of remdesivir were studied in COVID-19 patients from intensive care units (ICUs) and infectious disease wards (IDWs). The approximately same level of increase in aminotransferase was observed in both groups (ICU: 44.4%; IDW: 41.2%); however, the level of bilirubin increased more in the ICU group than in IDW individuals, indicating that the difference in enzyme levels may be related to the difference in symptomatic severity in patients[59]. In another case, an acute rise in ALT was noted after the start of remdesivir for 2 d and was immediately reversed after stopping it. The patient developed hepatotoxicity, which was proposed to be due to a drug-drug interaction of remdesivir and P-glycoprotein inhibitors. Hence, it was suggested to use the drug with caution[60]. Remdesivir-associated liver failure improved in a study reported by Carothers *et al*[61], suggesting that the use of acetylcysteine could be beneficial. However, there is a limitation in data regarding the management of acetaminophen-associated liver failure with acetylcysteine.

Similarly, favipiravir (avigan) is also considered for the treatment of SARS-CoV-2 infection. It is a broad-spectrum antiviral medicine that was initially administered for the treatment of influenza in Japan. Favipiravir is a prodrug that is taken up by viral RNA polymerase as a purine nucleotide after being intracellularly phosphorylated to form the active metabolite (favipiravir ibofuranosyl-5′-triphosphate), effectively inhibiting RNA-dependent RNA polymerase. It has been effective against RNA viruses such as West Nile virus, yellow fever virus, foot-and-mouth disease, Ebola, and Lassa virus[62]. In a case study, favipiravir was found to cause cholestatic liver injury. The authors suggested that liver injury developed due to the use of antibacterials followed by a high dose of favipiravir (6000 mg on the 1st d and 2400 mg for 14 d), which worsened liver function with elevated transaminase and total bilirubin levels[63].

A recent study comparing patients in the favipiravir group to those in the control group receiving lopinavir/ritonavir 400 mg/100 mg twice daily for 14 d plus aerosolized interferon-α by inhalation (5 million U twice daily) showed that the favipiravir group significantly reduced the amount of time needed for viral clearance (median 4 d *vs* 11 d) while also experiencing fewer side effects[64]. Additionally, teratogenicity (abnormal fetal development), hyperuricemia[50], diarrhea, and neutropenia are the known side effects of this drug[65]. Although the dose regimen for clinical trials or experimental drugs used for COVID-19 patients is aided by information from the treatment of influenza, more clinical testing is necessary to determine the exact effectiveness of favipiravir[65].

Guaraldi *et al*[66] in a retrospective cohort study found that tocilizumab (an IL-6 receptor antagonist) does not have any harmful effects on the liver when administered to 1351 COVID-19 patients. However, serum transaminase levels were elevated up to 40 fold[67]. Similarly, a significant correlation was later found between the administration of lopinavir/ritonavir, hydroxychloroquine, remdesivir, and tocilizumab, leading to liver damage in 1827 patients[68]. Other drugs, including hydroxychloroquine and azithromycin, are known to be used for SARS-CoV-2 infections leading to liver damage[69].

Most of these drugs also inhibit liver transporters including ABCB11/BSEP, ABCC2/MRP2, SLC47A1/ MATE1, ABCC3/MRP3, ABCC4/MRP4, SLC22A1/OCT1, SLCO1B1/OATP1B1, SLCO1B3/OATP1B3, and SLC10A1/NTCP. Since these transporters play a significant role in the clearance of endobiotics and other toxic compounds, their inhibition may affect liver functioning and cause alterations in the GI tract and kidney damage[1].

Since inflammatory cytokines are produced in response to COVID-19 infection, excessive cytokine (cytokine storm) causes septic shock, tissue damage, and organ failure. The microbial imbalance found in the blood analysis of COVID-19 patients was also linked to elevated levels of inflammatory cytokines and blood markers, such as C-reactive protein and specific enzymes, as a result of tissue damage[20]. The gut dysbiosis-associated hepatic inflammation led to severe symptoms of COVID-19 and increased mortality. Therefore, incorporation of the drug-induced gut dysbiosis and its connection to hepatic injury could better elucidate the gut-liver-bidirectional-axis association with COVID-19 severity. A precise understanding of interaction between gut microbiota and liver physiology will facilitate the development of targeted therapeutics to improve the condition of COVID-19 patients.

GI MANIFESTATION AND GUT DYSBIOSIS (METABOLIC DYSFUNCTION)

Studies have suggested that altered gut microbiota (dysbiosis) can play a significant role in immune-

mediated inflammatory diseases[70]. Similarly, dysbiosis of the gut microbiota might determine the clinical outcome of patients with underlying comorbid illnesses such as type 2 diabetes, hypertension, and obesity in COVID-19[71]. A potential role of gut microbiota in overall pathogenesis and outcomes is implied by the fact that gut microbial diversity generally decreases with age and that COVID-19 severity and fatality increase in older individuals. The impact of this disease can be minimized by improving the gut microbiota profile *via* personalized nutrition and supplements that improve immunity in older patients and immunocompromised individuals[72].

It has further been suggested that patients with COVID-19 have compromised gut microbiota, which has well-known immunomodulatory potential. Blood and stool samples were collected from 100 COVID-19 patients in a two-hospital cohort study. A significant alteration in gut microbial composition was found in COVID-19 patients compared to healthy individuals. Gut commensal microbiota with known immunomodulatory potential, such as *Bifidobacteria*, *Faecalibacterium prausnitzii*, and *Eubacterium rectale,* decreased in number and remained low even when the samples were collected until 30 d after recovery from COVID-19 infection. Moreover, blood markers, including AST, C-reactive protein, lactate dehydrogenase, and GGT, were elevated in this perturbed composition, which also showed stratification with disease severity[20]. Since the digestive and respiratory systems have an impact on each other *via* the gut-lung axis (common mucosal immune system), it is believed that improved GI ecology will have a positive impact on COVID-19 patients[73].

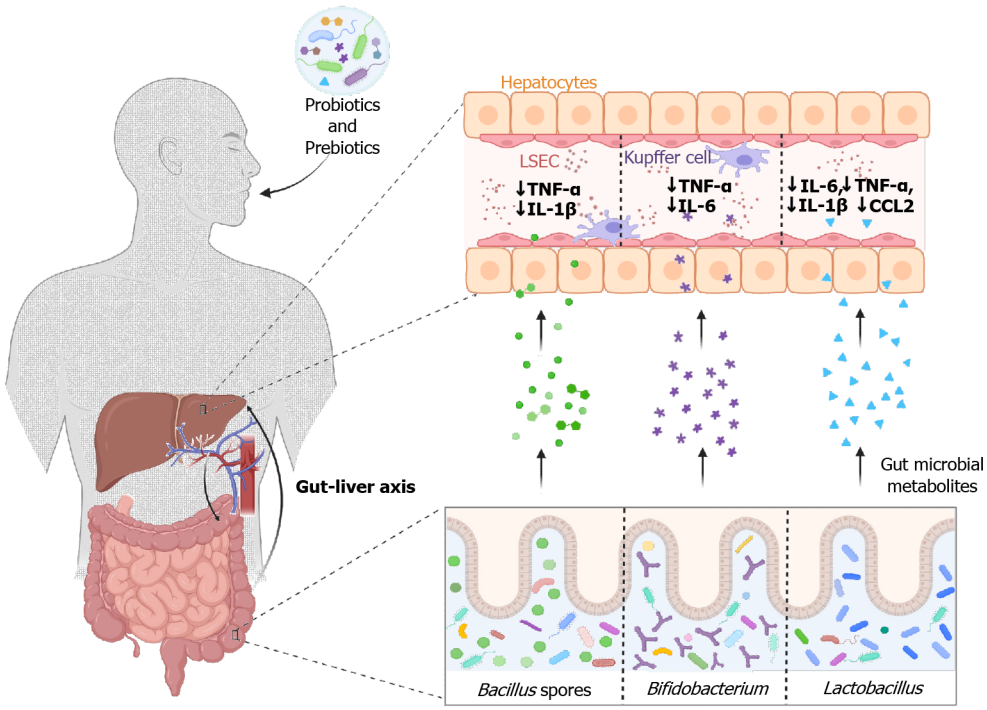
Although a small case series from China suggested that COVID-19 patients had less *Lactobacillus* and *Bifidobacteria* during microbial dysbiosis, no conclusive research linked the intestinal microbiota to COVID-19 at that time[74]. However, the SARS-CoV-2 receptor ACE2 was found to control intestinal microbial homeostasis *via* amino acids in a previously reported study[75]. Gut microbiota are known to produce SCFAs by fermentation. Predominant gut bacteria [*Ruminococcaceae* (*cluster IV*) and *Eubacterium* (*cluster XIVa*) in order *Clostridia* and phylum *Firmicutes*] produce SCFAs including acetate, propionate, and butyrate, which are frequently metabolized[76]. SCFAs that remain undigested further promote the formation of naïve CD4+ T cells, which mainly aid in controlling the level of lymphocytes in bone marrow and peripheral blood circulation (Figure 1). Consequently, gut microbial homeostasis is disturbed, ultimately compromising the immune system.

Increased levels of cytokines and inflammatory cells during COVID-19 infection are linked to sepsis and acute respiratory distress syndrome (ARDS). Inflammatory cytokines [IL-6, IL-8, IL-10, and tumor necrosis factor α (TNF-α)] were found to be high in number, leading to ARDS and multiple-organ dysfunction[77,78]. Butyric acid produced by intestinal bacteria is known to reduce cytokine storm[79]. Thus, the gut microbiota could help in reducing the prevalence of ARDS and sepsis, which are major mortality risks in COVID-19. Moreover, some researchers think that sepsis and abnormalities of the gut microbiota should be promoted together[80].

POTENTIAL THERAPEUTICS: PROBIOTICS AND PREBIOTICS

Considering the link between gut dysbiosis due to cytokine storm and COVID-19 severity, modulation of the gut microbiome holds great therapeutic potential for disease modification (Figure 2). However, there is currently no microbiota-directed therapy that has been shown to be effective in preventing the development or progression of COVID-19. Nevertheless, scientists are raising concerns about the health benefits and disease prevention properties of diet and gut microbiota during the course of infection. Growth of *Bifidobacterium* and *Lactobacillus* spp. in the human gut is promoted by plant-based fibers, which also help reduce harmful microbiota (*Clostridia*)[81]. Since microbial SCFAs are produced by the fermentation of dietary fibers and have anti-inflammatory effects, fiber intake can improve the host immune system[82]. When tested in mouse models, a fiber-rich diet promoting SCFA was found to increase immunity against allergic inflammation in the lungs, whereas a low-fiber diet with low SCFA levels increased allergic airway disease[83,84]. Studies have verified that the use of whole-grain fiber can reduce the mortality rate in various respiratory diseases[85].

Similarly, oral administration of probiotics alters the composition of the gut microbiota once it reaches the intestine[81]. Several studies have shown that the consumption of probiotics (beneficial bacteria) changes the local and systemic inflammatory balance, which in turn reduces respiratory infections and other extra-intestinal illnesses. When *Lactobacillus gasseri* SBT2055 was administered orally as a probiotic in mice, the inflammatory response against respiratory syncytial virus infection in the lungs was repressed. Levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β, and chemokine ligand 2) significantly decreased and were maintained at equivalent levels compared to control mice [86]. Furthermore, the cellular immunity of 30 elderly volunteers was boosted when they took *Bifidobacterium lactis HN019*[87]. Similarly, placebo-controlled clinical trials using the probiotic *Bacillus subtilis* and *Enterococcus faecalis* were effective and safe ways to prevent ventilator-associated pneumonia and gastric colonization of potentially pathogenic microorganisms[88]. Probiotics seem to be among the most suitable, efficient, and potentially safe strategies if dysbiosis is indeed involved in the pathogenesis of severe COVID-19. In fact, the National Health Commission (China) suggested the use of probiotics for maintaining gut microbial homeostasis and preventing secondary bacterial infections[89].



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Figure 2 Modulation of gut microbiota and its role in the gut-liver axis during coronavirus disease 2019. Probiotics and prebiotics could be used as potential therapeutics to lower coronavirus disease 2019 symptom severity by producing various bioactive metabolites, which are absorbed into the liver mainly *via* the hepatic portal vein, for regulation of hepatic function by reducing inflammatory cytokines. CCL2: Chemokine (C-C motif) ligand 2; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; LSEC: Liver sinusoidal endothelial cells; TNF-α: Tumor necrosis factor-α.

Since drug-induced liver damage is one of the major outcomes of COVID-19 infections, scientists are focusing on possible strategies to mitigate liver damage. As discussed, hepatotoxicity is most likely induced by chemical exposure, disrupted intestinal microbiome, gut mucosal barrier damage, and systematic immune activation. However, many preclinical studies have shown that prebiotic and probiotic supplementation could improve drug-induced liver injury[90]. Pretreatment with *Lactobacillus reuteri* DSM 17938 was performed on rats after undergoing a model of liver failure. Not only did it lower serum ALT, AST, GGT, IL-1, IL-2, IL-18, macrophage colony-stimulating factor, and macrophage inflammatory protein 3α levels, but it also improved histological abnormalities in the terminal ileum and liver caused by d-galactosamine[91].

Similarly, the hepatotoxic effect of acetaminophen was observed to be reduced using probiotic Mega Spore Biotic TM, which is a *Bacillus* spore-based probiotic. It also decreased proinflammatory cytokines such as TNF-α and L-1β and reduced hepatocyte necrosis[92]. Acetaminophen is a widely used antipyretic and has adverse effects in COVID-19 patients[93]. Moreover, the use of *Bifidobacterium adolescentis* CGMCC15058 in rats with liver failure was reported to have therapeutic effects with reduced levels of inflammatory liver cytokines such as TNF-α and IL-6[94]. Amplicon sequencing revealed the loss of potential SCFA-producing gut microbiota, such as ASV0AKS_*Oscillibacter*, ASV009F_*Anaerofustis*, ASV02YT_*Blautia*, ASV07LA_*Blautia*, and ASV0AM6_*Eubacterium hallii* in post-acute COVID-19 syndrome. These gut microbial species could be elevated using a high-fiber formula. Clinical parameters such as alkaline phosphatase, AST, ALT, albumin, and total bilirubin returned to normal levels after a high-fiber diet, leading to improved post-acute COVID-19 GI symptoms and liver function[95].

CONCLUSION

The gut microbiota play an important role in maintaining human health. Irrespective of the poor understanding of the connection between the gut and drug-induced hepatic injury mechanisms, the gut microbiota poses a significant role in liver protection through different pathways seems to be critical. The use of antiviral agents in SARS-CoV-2 patients results in gut dysbiosis that may predispose patients to severe COVID-19, as intestinal permeability and bacterial products spilling out enhanced by increased proinflammatory cytokine due to liver damage led to the severity of symptoms. It is therefore crucial that we explore potential preventive and therapeutic targets, such as probiotics and dietary interventions for gut rebiosis. Different probiotics using diverse prebiotics produce a variety of hepatic protective bioactive metabolites that could mitigate drug-induced liver damage during COVID-19. We

believe that the risk of drug-induced liver injury could be minimized by boosting hepatic function *via* rebuilding the dysbiotic intestinal environment with probiotics and prebiotics. The targeted intervention of gut microbiota may regulate the intestinal microbial community and thus manage liver injury. Moreover, we suggest well-planned experiments on animal models and clinical trials to understand the interactions between gut microbes and liver diseases to use this approach comprehensively.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Locked nucleic acid real-time polymerase chain reaction method identifying two polymorphisms of hepatitis B virus genotype C2 infections, rt269L and rt269I

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Abstract

BACKGROUND

The presence of two distinct hepatitis B virus (HBV) Pol RT polymorphisms, rt269L and rt269I, could contribute to the unique clinical or virological phenotype of HBV genotype C2. Therefore, a simple and sensitive method capable of identifying both types in chronic hepatitis B (CHB) patients infected with genotype C2 should be developed.

AIM

To develop a novel simple and sensitive locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method capable of identifying two rt269 types in CHB genotype C2 patients.

METHODS

We designed proper primer and probe sets for LNA-RT-PCR for the separation of rt269 types. Using synthesized DNAs of the wild type and variant forms, melting

temperature analysis, detection sensitivity, and endpoint genotyping for LNA-RT-PCR were performed. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

RESULTS

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients. When the results were compared with those obtained by the direct sequencing protocol, the LNA-RT-PCR method showed the same results in all but one of 87 positive detected samples (98.9% specificity).

CONCLUSION

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Key Words: Hepatitis B virus; Genotype C2; Polymerase; rt269; Locked nucleic acid-real time-polymerase chain reaction; Chronic hepatitis B

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Core Tip: Hepatitis B virus (HBV) genotype C2 infections have distinct clinical or virological traits, including a higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. We recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, contributed to unique traits of HBV genotype C2. Here, instead of time- or labor-consuming direct sequencing, we developed a new locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method for the separation between rt269L (L1 and L2) and I type from Korean chronic hepatitis B patients of genotype C2. The newly developed LNA-RT-PCR could be effectively used for the understanding of epidemiology and disease progression in genotype C2 endemic areas.

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INTRODUCTION

Although vaccines and therapeutic agents are currently available against hepatitis B virus (HBV), HBV infection is still a high-risk global health issue. More than 350 million people are chronically infected, and approximately 786000 patients die annually worldwide due to HBV-related diseases, including cirrhosis and hepatocellular carcinoma (HCC)[1,2].

HBV belongs into hepadnaviridae and is an enveloped and partially double-stranded DNA virus. Its genome is approximately 3.2 kb in length and contains 4 overlapping open reading frames: Surface antigens (S), core proteins (C), polymerase (Pol), and X proteins (X)[3]. The HBV reverse transcriptase can lead to HBV mutations of higher frequency than that of other DNA viruses due to its lack of proofreading ability[4,5]. This results in the failure of antiviral therapy with nucleos(t)ide analogs and liver disease progression *via* persistent infections[5-9]. According to the criteria of an 8% divergence in HBV genome sequences, HBV has been grouped into 10 genotypes as A-J[10-12]. A number of studies on HBV genotypes have reported that they play significant roles in the development of different disease profiles during chronic hepatitis B (CHB) infection as well as distinct geographic and ethnic distributions[13,14]. Of note, genotype C, particularly C2, *vs* genotype B showed a higher HBV replication capacity and higher tendency of chronicity and more frequently developed into liver cirrhosis (LC) and HCC in CHB patients of HBV endemic Asian nations, such as China, Japan and South Korea[11,15-19]. In addition, incomplete response to interferon (IFN) therapy and higher levels of mutations were also reported in genotype C2 infections[18,20-22]. However, thus far, which factor can explain several

distinct characteristics in clinical and virological aspects found in genotype C2 infections remains elusive.

As one likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, that are found only in HBV genotype C could affect viral phenotypes and clinical outcomes and cause worse responses to IFN therapy in genotype C2 infections. In particular, we showed that the wild rt269L type infection that is distinct in genotype C *vs* the rt269I type is more strongly related to higher HBV replication and hepatitis B e antigen (HBeAg) positive serostatus, which are two distinct traits of genotype C infections[23-25]. This suggests that the presence of RT polymorphisms, particularly the wild rt269L type, could at least partly contribute into clinical or virological traits that are distinct in genotype C infections. However, our previous study has limitations in exploring the distribution of rt269 polymorphisms in CHB patients due to use of a conventional nested polymerase chain reaction (PCR) based direct sequencing protocol, which could underestimate genuine HBV quasispecies in patient sera[23]. A locked nucleic acid (LNA) is a nucleic acid analog containing a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon[26,27]. The real time PCR method using a LNA-based probe capable of improving the hybridization affinity for complementary sequences shows strong mismatch discriminatory power[28,29]. Therefore, without the application of nested PCR, it could discriminate HBV mutations from CHB patients with high sensitivity and specificity.

Therefore, in this study, for the first time, we sought to develop a novel simple and sensitive locked nucleotide probe (LNA probe)-based RT-PCR (LNA-RT-PCR) method that is capable of separating two different rt269 polymorphisms, the wild-type rt269L (CTC/A) and rt269I type (ATC), in CHB patients of genotype C2.

MATERIALS AND METHODS

Patient sera, HBV DNA extraction and genotyping

For this study, serum samples from 94 patients who visited Seoul National University Hospital (2005-2007), met the inclusion criteria of hepatitis B surface antigen (HBsAg) positivity and HBV DNA positivity (for more than 6 mo), and were lamivudine, adefovir dipivoxil, entecavir, telbivudine, tumor necrosis factor, and peg-IFN treatment-naïve were used. All patients had negative tests for hepatitis C virus, human immunodeficiency virus and markers for coexisting autoimmune liver disease and did not have an alcohol or drug addiction. HBV DNA was extracted from 200 μL of serum samples using the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Hilden, Germany). To analyze the genotyping, a nested PCR-based sequencing protocol targeting partial HBsAg sequences was used as previously described [30]. This study was approved by Seoul National University Hospital (IRB-1012-131-346).

Synthesis of positive control DNAs for variants at the HBV rt269 codon

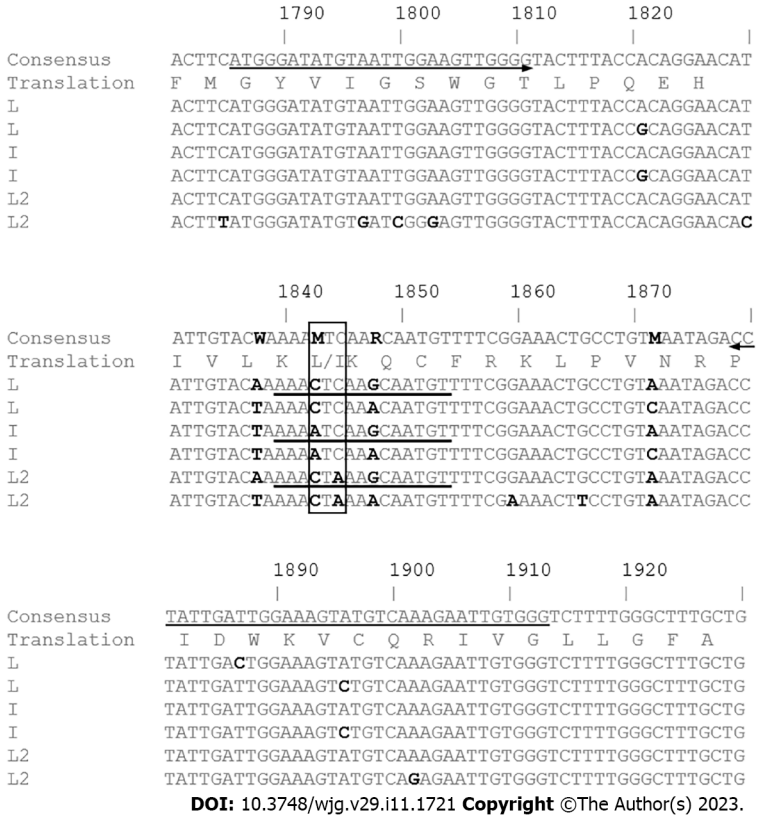
We prepared six positive control DNAs for L1 [CTC, wild type (WT)] and the variants I (ATC) and L2 (CTA) at the HBV rtL269I locus. The DNAs were synthesized based on the HBV C2 polymerase sequence by Integrated DNA Technologies, Inc. They were 473 bp long and included the three variant sequences, with one of the 'A/G' polymorphisms near the variant sequence (Figure 1, Supplementary Table 1). These were used for the development of the methods for the application of LNA real-time PCR to a rapid differential and quantitative identification of the WT and variants. We used these DNAs to intentionally mix DNA templates with WT control DNA and variant control DNA in different ratios in a range of amounts to mimic clinical samples. We also used positive controls for melting temperature (T_m) analysis, detection sensitivity, and endpoint genotyping and for the construction of quantification standard graphs for LNA-RT-PCR to estimate the quantity of HBV WT and variant DNA in clinical samples.

Primer and LNA probe design

Primers were designed using LightCycler Probe Design Software 2.0 (LC PDS 2.0) Version 1.0.R.36 (Roche). The primers were designed to have high melting temperatures (> 65 °C) and to be highly conserved in the target DNA region of HBV. We used LC PDS (version 2.0) software for the probe design and referred to the design guidelines of the LNA manufacturer (Integrated DNA Technologies). The potential presence of cross-complementarities among all the primers and LNA probes was checked by using LC PDS 2.0 software. The LNA probes were purchased from Integrated DNA Technologies, and primers were purchased from Macrogen.

RT-PCR

A LightCycler Version 96 system (Roche) was used for LNA-RT-PCR, and three channels were used for the experiment. An optimal reaction mixture was established for the sensitive and specific detection of target sequences. A 10-μL reaction mixture was prepared for each sample as follows: 1 μL PCR buffer for *Taq* (Ex *Taq* HS, Takara), 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphate mixture



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Figure 1 Primer and locked nucleic acid probe positions designed for the detection of three genotypes of polymorphisms in the rt269 codon, 'L1', 'I' and 'L2'. Arrows indicate the primer positions. Underlines indicate the probe positions. The numbers designate the nucleotide position on the hepatitis B virus *P* gene sequence. Boldface bases denote the different bases. The box represents the codon and amino acid sequences of rtL269 variants. This single nucleotide difference is the basis of their discriminative identification by locked nucleic acid probes in this study. The amino acid sequence is shown as one-letter amino acid symbols.

(Takara), 0.2 μM forward primer, 0.8 μM reverse primer, 0.4 μM LNA FAM probe (L_CTC), 0.4 μM LNA Hex probe (I_ATC), 0.2 μM LNA Cy5 probe (L2_CTA), 0.25 u Ex *Taq* HS (Takara), 1 mg/mL bovine serum albumin (Ambion, ThermoFisher), 2 μL template DNA, and PCR-grade water (Roche). The cycling conditions were as follows, with default ramping speed rates if not specified: 60 s at 95 °C; four cycles of 10 s at 95 °C, 10 s at 58 °C, and 25 s at 72 °C with a 2.2 °C/s ramp; 46 cycles of 10 s at 95 °C, 10 s at 58 °C (with a single fluorescence acquisition), 25 s at 72 °C with a 2.2 °C/s ramp, and melting-curve analysis with 10 s at 95 °C, 60 s at 53 °C, and 1 s at 80 °C with a 0.08 °C/s ramp under continuous fluorescence acquisition at a rate of 4 readings/°C.

Identification of the WT and variant forms

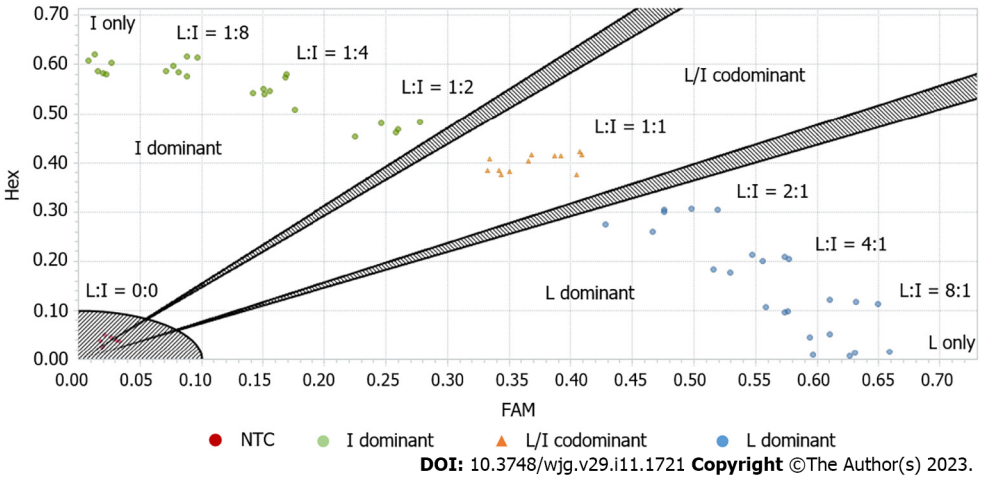
Identification of the WT and variant forms 'I' and 'L2' at the rt269 codon in a sample was performed based on the three different LNA probe-specific T_m measurements at their own specified channels. To establish the diagnostic T_m range for the WT and variant forms, the control DNAs of the WT form, the variant forms and their mixtures at a variety of ratios were tested to observe melting peak formation and measure the specific T_m values for the WT and variant forms.

Construction of standard quantification curves

Six types of standard quantification curves for the WT and variant forms were generated with known amounts of positive control DNAs for their application to the estimation of the amount of the target DNAs in unknown samples. The standard curves were produced by duplicate LNA real-time PCR for each target DNA with known amounts (4.0E + 08 to 4.0E + 01 copies) of control DNAs. The R^2 correlation for all the standard curves was greater than 0.99. The limit of detection and limit of quantification of the WT and variant forms were determined among the series of diluted copies. These standard curves were applied to the quantification of DNA samples in a pure form and dominant type of variants in a mixed form.

Construction of standard genotyping plots to determine a dominant type in a mixture sample

To determine a dominant type of rtL269I variant in a mixture of a sample, standard genotyping plots were constructed using LNA real-time PCR with positive control DNA mixture sets in various ratios



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Figure 2 Differentiation of dominant hepatitis B virus rtL269 genotype variants using an endpoint genotyping method (LC96 software). L plus I genotype mixtures were prepared with known amounts of the genotypes in various ratios. I-dominant mixtures were positioned closer to axis Hex with higher Hex-fluorescence values, whereas L-dominant ones were located closer to axis FAM with higher FAM-fluorescence values. NTC: Nontemplate control.

and the endpoint genotyping tool of LC 96 system software. These plots were based on the endpoint fluorescence (EPF) values at the two channels for comparison. These plots were applied to determine the dominant type in the clinical samples (Figure 2).

Application of LNA-RT-PCR to clinical samples

The DNA of a total of 94 human sera was tested for the identification of the WT and 'I' and 'L2' variant forms of the HBV RT gene by LNA-RT-PCR. The quantification cycle (Cq), EPF, and T_m produced by the WT- and variant-targeting LNA probes with sample DNA were measured. Identification of the WT and variant forms was determined by comparing their T_m values obtained from their specific channel (FAM for WT, Hex for 'I', and Cy5 for 'L2') with their diagnostic T_m ranges obtained from standard assays.

Comparison of LNA-RT-PCR and direct sequencing for identification of WT and variant DNA

A total of 94 clinical samples were tested for the comparison of the LNA-RT-PCR method and directing sequencing method in the accurate identification of the rt269 variant and WT DNA. Direct sequencing was performed using the same primer sets producing the 128-bp LNA-RT-PCR amplicon.

RESULTS

Primer and probe design for LNA-based RT-PCR

First, we investigated the full-length HBV reverse transcriptase sequences from 131 treatment-naïve Korean patients chronically infected with HBV genotype C2 (GenBank No CH patients (GenBank Nos: KX264864-KX264922) and HCC patients (GenBank Nos: KX264792-KX264863)[30]. SeqMan II software Version 5.03 (DNASTAR) was used to search for appropriate primer sequences for LNA-based RT-PCR that are highly conserved to first obtain the shortest possible amplification product of the rt269 codon for efficient PCR (Figure 1).

We found three distinct sequence types in the rt269 codon from 131 patients, two types in rt269L, CTC (designated L1) and CTA (designated L2), and one rt269I type, ATC (designated I). Therefore, we designed three different LNA probes for specific simultaneous detection in a single reaction of the 'L' (WT), 'I', and 'L2' variants of HBV. The sequences of primers and LNA probes are shown in Table 1 and Figure 1.

Determination of the diagnostic T_m range for the identification of the WT and variant forms

Identification of the three sequence types, "L1", "I" and "L2", was accomplished by LNA-RT-PCR melting curve analysis by observation of their melting peak formation and their specific T_m measurement at their specified channel (Table 2, Figure 3). LNA-RT-PCR with samples of WT control DNA (n = 68) in amounts ranging from 4.0E + 00 to 4.0E + 08 copies resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the FAM channel in all the tested WT control DNA samples with T_ms of 62.4 ± 0.4 °C for 'L' and 58.0 ± 0.2 °C for 'L" was observed, but no significant melting peak formation at the other channels (Hex and Cy5) was observed. LNA-RT-PCR with samples of the 'I' positive control DNA (n = 76) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation in the Hex channel, 60.2 ± 0.7 °C for 'I' and 56.6

Table 1 Primers and locked nucleic acid probes developed for the identification of the hepatitis B virus L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Primer/probe	Sequence (5' to 3')[1]	T_m (°C)[2]	Target	CH
Primers (product: 128 bp)				
Forward	ATGGGATATGTAATTGGAAGtTGGGG	65-67	HBV P gene	
Reverse	CCCACAATTCttTGACATACTTTCCAATCAATAGG	67-69	HBV P gene	
LNA Probes				
L_CTC	5′ 6-FAM-AAA+C+T+CAAR+CA+ATGT - 3′ IABkFQ	61-64	L (WT)	FAM
I_ATC	5′ HEX-AAA+A+T+CAAR+CAA+T+GT - 3′ IABkFQ	61-64	I	HEX
L2_CTA	5′ CY5-AAA+C+T+AAAR+CAA+T+GT - 3′ IABkFQ	60-63	L2	CY5

[1]Locked nucleic acid nucleotides are written +A, +C, +T or +G.

[2]Primer T_m was calculated by using LC PDS software version 2.0, and probe T_m was calculated by https://www.exiqon.com/ls/pages/exiqontmpredictiontool.aspx.

T_m: Melting temperature; CH: Channel; HBV: Hepatitis B virus; WT: Wild type; LNA: Locked nucleic acid.

± 0.2 °C for 'I'', was observed, but no significant melting peak formation in the other channels (FAM and Cy5) was observed. LNA-RT-PCR with samples of the 'L2' positive control DNA (n = 52) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the Cy5 channel, 64.6 ± 0.1 °C for 'L2' and 61.2 ± 0.2 °C for 'L2'', was observed, but no significant melting peak formation at the other channels (FAM and Hex) was observed.

LNA-RT-PCR with samples (n = 320) of the 'L' WT positive control DNA plus 'I' control DNA or 'L2' plus 'I', mixed in different ratios (1:1, 1:2, 1:4, 1:8, 2:1, 4:1, and 8:1) in amounts ranging from 4.0E + 01 to 4.0E + 08 copies resulted in a nearly 100% positive detection rate (only three samples undetected in the smallest amount of DNA) for both the variant and WT DNA. A distinct melting peak formation at the FAM, Hex, and Cy5 channels in all the mixed DNA samples with detectable T_ms was observed. The measured T_ms were shifted slightly downward from the range of the T_ms measured only with nonmixed DNAs, as shown in Table 1. These slight changes did not affect the identification of the sequence types in the samples.

Application of LNA-RT-PCR to clinical samples and comparison with the results of the direct sequencing protocol

Of the 94 clinical samples tested by our LNA-RT-PCR method, 87 samples (92.6% sensitivity) were positively identified as 'L1' (WT), 'I', and 'L2' variants in single or mixed forms. Among the positively identified samples (n = 87), all samples produced a distinct melting peak or peaks with a T_m or T_ms within the diagnostic T_m range for the WT form "L1" or the two variant forms "I" and "L2". Of the 87 positively detected samples, 63 (72.4%) and 24 samples (27.6%) were identified either singly or in a mixed manner, respectively. Of the 63 samples identified singly, the prevalence of the 'L1' type, 'I' type and 'L2' type was 82.5% (n = 52), 12.7% (n = 8) and 4.8% (n = 3), respectively (Table 3). Of the 24 mixed form samples (27.6%), the prevalence of samples with almost the same ratio of L1 and I (codominant cases) was 29.2% (n = 7). The prevalence of L1 (L1 + I or L1 + L2) and I dominant (L1 + I) cases was 54.2% (n = 13) and 16.7% (n = 4), respectively. Given that the dominant cases included the respective exclusive cases, of the 87 positively detected samples, the prevalence of L, I and coinfection with L and I was 78.2% [n = 68, L1(65) + L2(3)], 13.8% (n = 12), and 8.0% (n = 7), respectively. PCR direct sequencing using the same primer set used in the LNA-RT-PCR method enabled the successful separation between the L1, L2 and I sequence types in all 94 clinical samples (100% sensitivity). Comparison between results obtained by both direct sequencing and LNA-RT-PCR protocols showed that of the 87 samples identified by LNA-RT-PCR, all (86 samples, 98.9% specificity) but one sample (SNU3-479) produced completely identical results between the two protocols (Figure 4). A mismatched sample was identified as I dominant (L1:I = 1:4) by LNA and exclusive I type by the direct sequencing protocol. The distinct results between both protocols may be due to the difference in sensitivity between the protocols. All seven samples not detected by the LNA-RT-PCR method were demonstrated to have mutations in their respective probe binding sequences by a direct sequencing protocol, which could interfere with normal LNA-RT-PCR (Table 4).

Table 2 Measurement of melting temperatures of the L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Genotype of positive control DNA [copies, (4.0E + 00)-(4.0E + 08)]	Target sequence[1]	Measured T_m (°C) in channel[2]								
		Min[3]	Max[3]	mean ± SD (detection)[3]	Min[4]	Max[4]	mean ± SD (detection)[4]	Min[5]	Max[5]	mean ± SD (detection)[5]
L (n = 34)	AAA**CTC**AAGCAATGTT	61.6	63.1	**62.4 ± 0.4** (34, 100%)	-	-	- (0, 0%)	55.2	56.2	55.8 ± 0.2 (26, 76.4%)
L′ (n = 34)	AAA**CTC**AAACAATGTT	57.8	58.7	**58.0 ± 0.2** (34, 100%)	-	-	- (0, 0%)	-	-	- (0, 0%)
I (n = 42)	AAA**ATC**AAGCAATGTT	-	-	- (0, 0%)	59.0	61.6	**60.2 ± 0.7** (42, 100%)	-	-	- (0, 0%)
I′ (n = 34)	AAA**ATC**AAACAATGTT	-	-	- (0, 0%)	56.3	57.1	**56.6 ± 0.2** (34, 100%)	-	-	- (0, 0%)
L2 (n = 34)	AAA**CTA**AAGCAATGTT	-	-	- (0, 0%)	-	-	- (0, 0%)	64.4	64.8	**64.6 ± 0.1** (26, 100%)
L2′ (n = 18)	AAA**CTA**AAACAATGTT	-	-	- (0, 0%)	-	-	- (0, 0%)	60.9	61.4	**61.2 ± 0.2** (18, 100%)

[1]Bold, target codon; underline, A/G polymorphism.
[2]Bold, target specific melting temperature.
[3]FAM.
[4]Hex.
[5]Cy5.
Bold words represent genotype-specific T_ms. T_m: Melting temperature; -: No significant melting temperature; SD: Standard deviation.

DISCUSSION

LNA-based RT-PCR assays have been widely applied to viral single-nucleotide polymorphism analysis as well as simple viral detection in clinical settings instead of the less sensitive traditional RT-PCR or nested RT-PCR assays prone that are to cross-contamination[31,32]. In particular, it has recently been reported that this method could successfully identify YMDD mutations of HBV from Korean patients with chronic HBV infections[30,33]. In the present study, we developed an LNA-RT-PCR assay using melting curve analysis for the identification of two polymorphisms within codon 269 of HBV Pol, rt269L and rt269I (three genotypes, rt269L1, rt269L2 and rt269I), with the advantages of easy performance and a low likelihood of cross-contamination. The clinical application of the LNA-RT-PCR assay was also compared in parallel with a direct sequencing protocol using clinical samples. Our data showed that the LNA-RT-PCR assay can separate the two polymorphisms in the rt269 codon of HBV Pol in clinical specimens with high sensitivity (92.6%, 87/94 samples) and specificity (98.9%, 86/87 samples) (Table 3). Of note, this assay can determine an almost exact ratio between two types within specimens from mixed cases (23/24 cases), suggesting its feasibility in the analysis of quasispecies distribution in mixed samples (Table 3, Figure 4).

Our LNA-based RT-PCR assays showed that the WT 'L1' type (n = 65, 74.7%) was found at the highest frequency in our cohort, followed by the 'I' type (n = 12, 13.8%) and 'L2' type (n = 3, 3.4%) (Table 3). This finding suggests that the 'L1' type is responsible for the majority of HBV infections in South Korea and that the WT form is prevalent in genotype C2 infections. Additionally, these findings suggest that the I type may be a variant of L1 rather than an independent polymorphism. Indeed, our

Table 3 Rates of positive detection of the hepatitis B virus L/I/L2 variants in a total of 94 clinical samples by locked nucleic acid real-time polymerase chain reaction

Type of detection	No. of samples	Percentage
Clinical samples	94	100
Single	63	67.0
L	55	58.5
I	8	8.5
Mixed	24	24.5
L + I (1:1)	7	7.4
L dominant	13	4.3
I dominant	4	12.8
Unidentified	7	7.4
Inconsistent with direct sequencing	1	1.1

Table 4 Samples which cannot be identified by locked nucleic acid real-time polymerase chain reaction assay

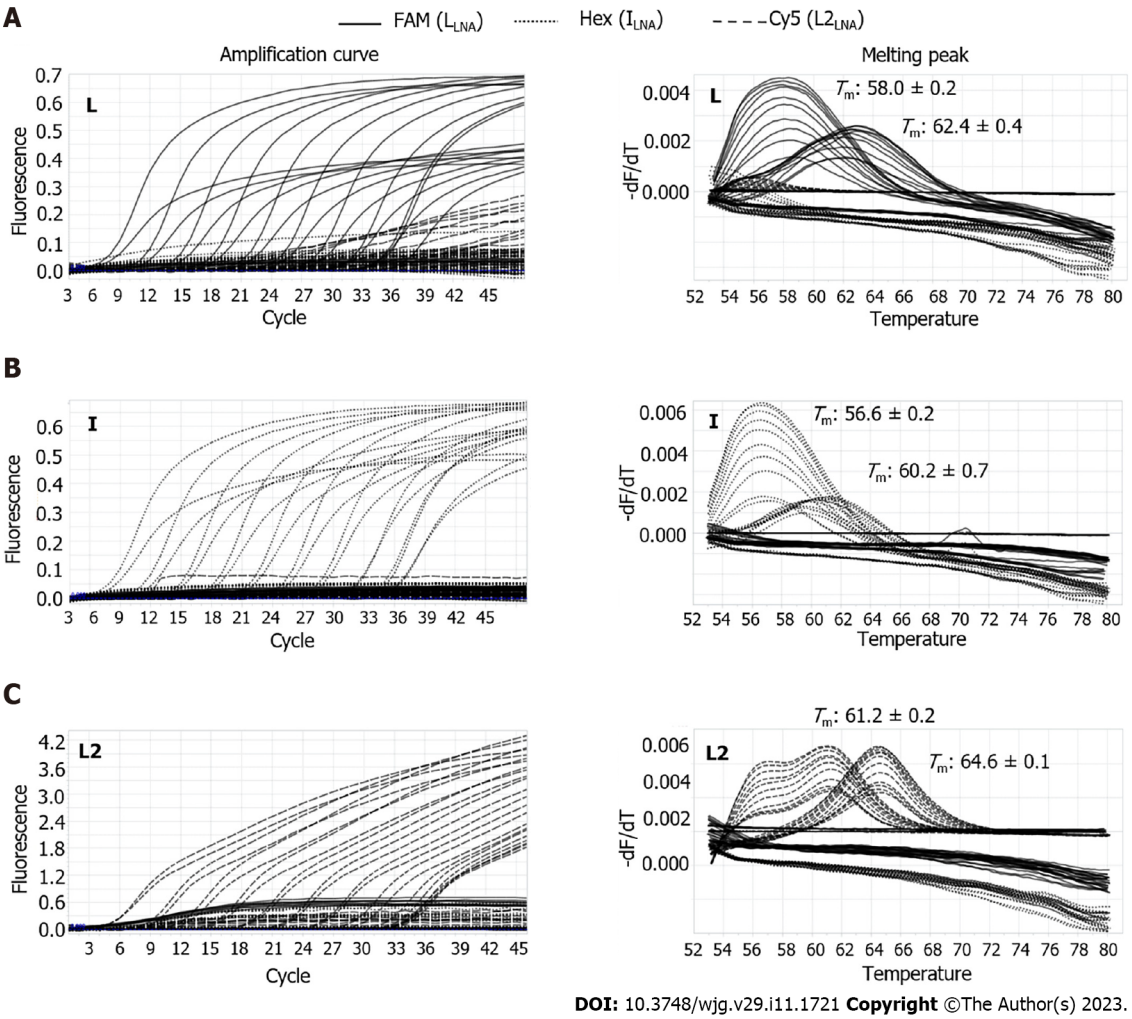
No.	Patients	Direct sequencing (AAACTCAARCAATGT)	Type	LNA-RT-PCR
1	SNU3 30 HCC	AAAATCAAGCA**C**TGT	I	Not detected
2	SNU3 70 HCC	AAAAT**T**AAGCAATGT	I	Not detected
3	SNU3 82 CH	AAAATCAAAC**T**ATGT	I	Not detected
4	SNU3 123 HCC	AAACT**T**AAGCAATGT	L	Not detected
5	SNU3 31 CH	AAAATC**C**AGCAATGT	I	Not detected
6	SNU3 355 LC	AAAAT**T**AAGCAATG	I	Not detected
7	SNU3 388 LC	AAACT**T**AAGCAATGT	L	Not detected

Bases in bold indicate the different ones from the target probe sequence. LNA-RT-PCR: Locked nucleic acid real-time polymerase chain reaction; HCC: Hepatocellular carcinoma; CH: Chronic hepatitis; LC: Liver cirrhosis.

previous study based on a direct sequencing protocol also showed that the 'L1' type *vs* the 'I' type is more closely related to higher HBV replication, higher HBsAg levels and HBeAg positive serostatus [23], suggesting that the majority of the 'L1' type infections in our cohort may be due to its enhanced viral infectivity. Therefore, it is tempting to speculate that the 'L1' type uniquely found in genotype C2 infections may contribute to some distinct traits of the genotype C2 infections, including an enhanced duration of the HBeAg-positive stage[34-36], higher infectivity[37,38] and a higher prevalence of occult infection *via* vertical transmission[33,39,40]. Since our LNA-based RT-PCR assays can identify L1 of higher infectivity and other variants (L2 or I type) related to disease progression from large serum samples without time-consuming or labor intensive sequencing procedures, it could help in the management or treatment of chronic patients in genotype C2 endemic nations, including China, Japan and South Korea.

In 7 (7.4%) of the 94 samples, despite successful amplification, our LNA-based RT-PCR assays failed to separate the two polymorphisms in the rt269 codon (Table 4). Comparison with the direct sequencing protocol revealed that all seven samples amplified but not identified by LNA-based RT-PCR assays had one more mismatch mutation that was different from the probe binding sequences. This was enough to interfere with normal detection due to the lower meting temperature than the respective probe. Therefore, in the samples amplified but not identified by our LNA-based RT-PCR assays, a further direct sequencing protocol should be recommended for the identification of the two polymorphisms.

A total of 24 (27.6%) of the 87 positively detected samples were identified in a mixed manner, and L1, in most cases of mixed infections, was dominant or codominant over I or L2. These findings further support our hypothesis that I or L2 may be a variant of the L1 type rather than an independent polymorphism. However, to clarify whether mixed infection in a patient is due to simple mutation of L1 to L2 or I type or superinfection of another type, further quasispecies analysis should be investigated in the future.



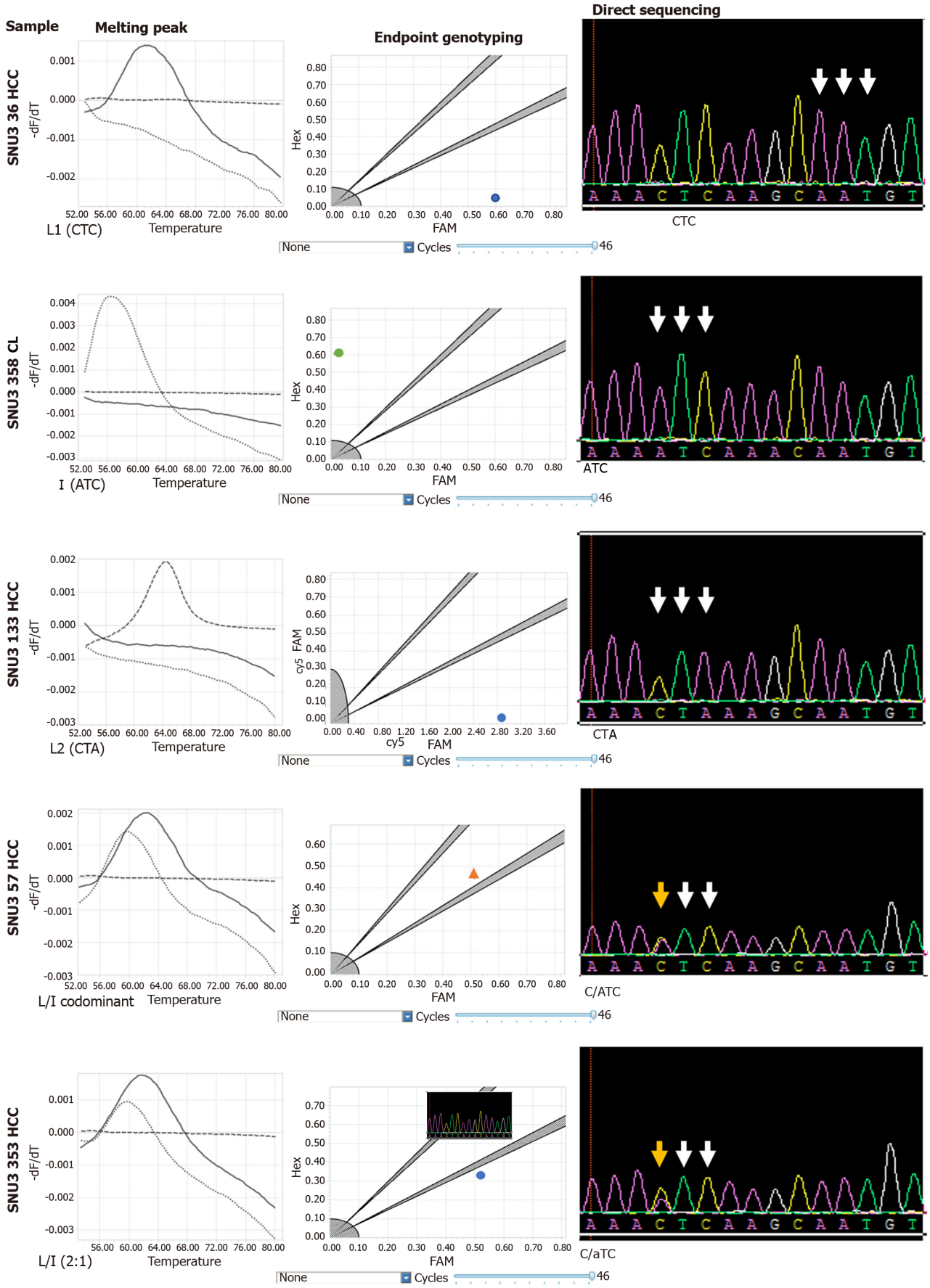
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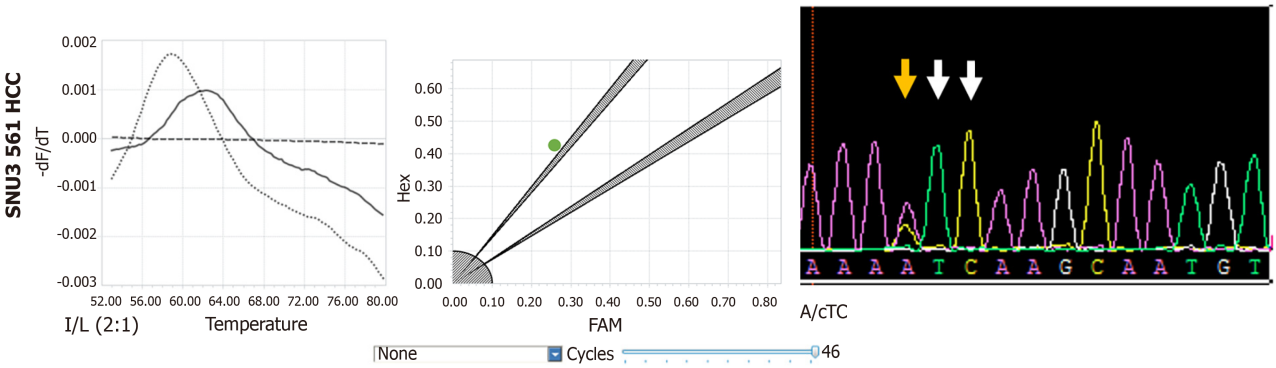
Figure 3 Multiprobe locked nucleic acid real-time polymerase chain reaction for discrimination among three types of polymorphisms in the rt269 codon. Amplification curves are shown on the left, and melting peaks are shown on the right. A: With L1 wild-type DNA templates, L1-type specific signals in the FAM channel (solid) were detected, showing their dominant amplification and distinct melting temperatures (T_m), with minimal cross signals of amplification and melting peaks generated by weak cross hybridizations of the other probes (I and L2), which were differentiated from the T_m values for I and L2 detection; B: For I variant-type DNA templates, I-type specific signals in the Hex channel (dotted) were detected, showing their exclusive amplifications and distinct T_m values, with no cross signals; C: For L2 variant-type DNA templates, amplification curves showed weak cross signals, but melting peaks were distinct with no cross signals.

The limitation of this study is that all the samples included were obtained from patients at the initial stage of drug use and are from one medical institution. To determine the exact clinical significance of L1, L2 and I infections or mixed infections in genotype C2-infected chronic patients, our LNA-based RT-PCR assays should be applied to a larger population-based cohort of multicenter registries in future studies.

CONCLUSION

In conclusion, our data showed that the LNA-RT-PCR method developed in this study can successfully identify two different polymorphisms, rt269L (L1 and L2) and rt269I, in the rt269 codon of HBV Pol from CHB patients with genotype C2 infections. The wildtype 'L1' form is more prevalent than the rt269I form in Korean CHB patients with genotype C2 infections, which is possibly due to its higher infectivity. Therefore, our LNA-RT-PCR method enables the separation of rt269 types and could be effectively used for a deeper understanding of epidemiology and disease progression in genotype C2 endemic areas.


Sample
Melting peak
Endpoint genotyping
Direct sequencing
SNU3 36 HCC
L1 (CTC)
Temperature
-dF/dT
Hex
FAM
CTC
None
Cycles
46
SNU3 358 CL
I (ATC)
ATC
SNU3 133 HCC
L2 (CTA)
cy5
CTA
SNU3 57 HCC
L/I codominant
C/ATC
SNU3 353 HCC
L/I (2:1)
C/aTC



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Figure 4 Confirmation of multiprobe locked nucleic acid real-time polymerase chain reaction identification results of hepatitis B virus rtL269 variants by direct sequencing. Nucleotide bases are shown in the parentheses. Lowercase letters represent the base present in a lower amount relative to the dominant variant. Bold indicates the dominant amino acids and bases. Arrows represent the codon sequence positions for leucine or isoleucine; yellow, mixed bases.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) genotype C infections has distinct clinical or virological traits including higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. As a likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I could contribute to unique traits of HBV genotype C.

Research motivation

For the identification between two rt269 types from chronic patients of genotype C2 endemic areas instead of time or labor consuming direct sequencing protocol, we sought to develop a novel simple and sensitive locked nucleotide probe based real-time polymerase chain reaction (LNA-RT-PCR) method capable of separating two rt269 types, rt269L type encoding leucine, 'L' (L1: CTC, L2: CTA) and rt269I type encoding isoleucine (ATC) from chronic hepatitis B (CHB) genotype C2 patients.

Research objectives

To develop a novel simple and sensitive LNA-RT-PCR method capable of identifying two rt269 types in CHB genotype C2 patients.

Research methods

We designed appropriate primer and probe sets for LNA-RT-PCR for the separation of rt269 types. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

Research results

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients.

Research conclusions

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Research perspectives

The newly developed LNA-RT-PCR method could identify three rt269 types, L1, L2 and I from CHB patients of genotype C2 with high-sensitivity and specificity. It could play a relevant role in the clinical management of CHB patients of genotype C2 infection.

FOOTNOTES

Author contributions: Kim K and Kim BJ contributed to study conception and design, and designed and performed experiments; Choe WH contributed to collection of clinical data; Kim K, Choi YM, Kim DH, Jang J, Choe WH, and Kim BJ contributed to data acquisition, data analysis and interpretation; Kim K, Choi YM, Choe WH, and Kim BJ contributed to writing of article, editing, reviewing and final approval of article.

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Informed consent statement: Informed consent was waived because of the retrospective nature of the study and the analysis used anonymous clinical data.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk factors predict microscopic extranodal tumor deposits in advanced stage III colon cancer patients

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Abstract

BACKGROUND

Colorectal cancer is a frequent cause of cancer-related mortality in patients with lymph node or distant metastases. Pericolonic tumor deposits (TDs) are considered prognostically distinct from lymph node metastases.

AIM

To investigate risk factors for extranodal TDs in stage III colon cancer.

METHODS

This was a retrospective cohort study. We selected 155 individuals diagnosed with stage III colon cancer from the database of the Cancer Registry of the Tri-Service General Hospital. The patients were allocated into the groups with/without N1c. Multivariate Cox regression analysis and Kaplan-Meier method were done. The primary outcomes investigate the association between the covariates and extranodal TDs, and prognostic significance of the covariates regarding the survival.

RESULTS

There were 136 individuals in the non-N1c group and 19 individuals in the N1c group. Patients with lymphovascular invasion (LVI) had a higher risk of TDs.

Overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively (P = 0.027). The N1c patients without LVI had higher overall survival than those who with LVI (7.73 years *vs* 4.42 years, P = 0.010).

CONCLUSION

Patients having stage III colon cancer with LVI have a higher probability of having TDs than those with stage III colon cancer without LVI. Stage III colon cancer patients with TDs and LVI could have poor prognosis and outcome.

Key Words: Colon cancer; Tumor deposits; Lymphovascular invasion; Risk factor

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Core Tip: Tumor deposits has been associated with poor outcome in patient with colorectal cancer. In our study, we investigated the risk factors predicting extranodal tumor deposits in stage III colorectal cancer patients according to the new American Joint Committee on Cancer TNM staging and helped pathologist not to miss the subgroup of N1c patients. Sincerely, we look forward to more robust therapeutic approach and closer survivorship planning for this subgroup of high-risk stage III colon cancer patients in the future.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer and the fifth most frequent cause of cancer-related mortalities worldwide[1]. The International Union Against Cancer/American Joint Committee on Cancer (AJCC) TNM classification has been utilized for cancer staging[2] and its function has aimed to determine patient care and treatment, as well as to predict cancer prognosis. The TNM staging system is revised every few years as knowledge of cancer continually expands[3].

A pericolonic tumor deposit (TD) is a perineural, peri- or intra-vascular tumor extending beyond the muscularis mucosae. It is different from lymph node metastases and should not be contemplated its prognostic value[4-7]. The disease-free survival influence of even small pericolonic TDs is significant[8], recommending that pericolonic TDs of all volumes should be contemplated clinically important[7,9,10].

The TNM classification of pericolonic TDs as lymph node metastases or sporadic tumor extensions is perhaps inaccurate. The quantity and greatest dimension of pericolonic TDs should be stated separately from lymph node metastases. In the seventh edition of the AJCC TNM staging system, TD was classified as pN1c in stage III colon cancer patients without lymph node metastasis[11].

Extranodal deposits are a different form of metastatic disease in patients with CRC. The relationship with vascular invasion and earlier development of metastases probably implies that a significant information of extranodal deposits may represent blood-borne spread. Some researchers have indicated worse prognosis of patients with TDs and have claimed that TDs should be categorized as M classification[6,9].

In this single-institution retrospective study, we investigate the risk factors predicting extranodal TDs in stage III CRC patients according to the new (eighth edition) AJCC TNM staging. In regard to possible poor prognosis of stage III CRC patient with factors predicting extranodal TDs, adjust the adjuvant chemotherapy regimens with target therapy or immunotherapy might be considered.

MATERIALS AND METHODS

Study design and population

All data were obtained retrospectively from the Cancer Registry database of the Tri-Service General Hospital (TSGH), Taipei, Taiwan. The cancer registry of the TSGH included 4067 patients with CRC from 2010 to 2016. The inclusion criteria were described as follows: (1) All the patients received colonoscopy with tumor biopsy and pathology proved malignancy; and (2) abdomen computed tomography or whole body positron emission tomography scan showed no distant metastasis. All patients received regular postoperative follow-up at our colorectal outpatient department. The exclusion

criteria were described as follows: (1) Lack of comprehensive data in long-term follow-up; (2) death within 30 d of surgery; (3) local excision or uncertain procedure; (4) synchronous tumors; and (5) rectal cancer or non-stage III colon cancer patients (Figure 1). A total of 155 individuals diagnosed with stage III colon cancer from a single institution of a medical center were enrolled. All patients signed informed consents, and the Institutional Review Board (IRB) of TSGH permitted our study (TSGHIRB No. C202005173). Our study was conducted only for medical research and was in accordance with the Declaration of Helsinki.

The definition of N1c

TDs, also called extranodal TDs, were defined as tumor cells in the pericolic or perirectal fat tissue without proof of residual lymph node tissue in the relevant lymphatic system of the primary tumor site [6]. According to the latest TNM 8th staging system announced in 2016, aiming to eliminate any lesion with identifiable structures pointing towards LN metastasis, extramural venous invasion or perineural invasion[12]. The hematoxylin eosin (HE) staining of extra-nodal TDs (N1c group) showed that there are nodules made up of tumor cells found in the structures near the colon that do not seem to be lymph nodes (Figure 2A). The HE staining of non-TDs (non-N1c group) showed that tumor cells revealed in regional lymph nodes (Figure 2B). A list of potential microscopic features that may be valuable to aid in the difference between tumor deposit and a positive lymph node was compiled and sent out for ranking with the virtual slides. The virtual slides were sent to three pathologists with a certain interest in gastrointestinal pathology for review. Each pathologist was asked to render an opinion of either tumor deposit or lymph node metastasis for each slide.

Covariates

The covariates comprised age, gender, body mass index (BMI), cigarettes smoking and alcohol consumption habits, hypertension (HTN), diabetes mellitus (DM), neutrophil-to-lymphocyte ratio (NLR)[13], carcinoembryonic antigen (CEA), CA 19-9, and tumor characteristics. The tumor characteristics included tumor location (right and left), T stage, mean tumor size, tumor type (ulcerative and polypoid), tumor grade (well, moderate, and poorly differentiated), epidermal growth factor receptor (EGFR) expression, and lymphovascular invasion (LVI). LVI was defined as tumor cells invading the lymphatic or blood vessels microscopically[14]. In our study, all specimens had been fixed immediately in formalin solution. For definite diagnosis, two pathologists resected our specimens for routine pathological examination independently by hematoxylin and eosin staining (H&E staining) and immunohistochemistry based on the eighth edition of the AJCC TNM system and the World Health Organization (WHO) criteria.

Statistical analysis

All data were analyzed using SPSS Statistics software (IBM Corp., Released 2016. IBM SPSS Statistics for MAC version 24.0. Armonk, NY, United States). Student's *t*-test was used to analyze quantitative variables in terms of the mean with SD. The chi-square test was used to analyze the qualitative variables in terms of frequency and percentage. Multivariate Cox regression analysis was performed to investigate the association between the covariates and extranodal TDs. The Kaplan-Meier method was used to calculate the overall survival and disease-free survival rates. Statistical significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

We included 155 individuals with stage III colon cancer in our study; their clinicopathological characteristics are shown in Table 1. There were 136 (88%) individuals in the non-N1c group and 19 (12%) individuals in the N1c group. The mean age of the N1c and non-N1c groups was 66.8 years and 65.2 years, respectively. N1c was observed mainly in male patients. The other characteristics showed no significant difference between the N1c and non-N1c groups, except for LVI ($P = 0.049$).

Risk factors of predicting extranodal TDs (N1c)

We investigated the association between the covariates and extranodal TDs using multivariate analysis (Table 2). There was no significant relationship between N1c and other confounding factors, such as age (HR = 1.03, 95%CI = 0.99-1.08), BMI (HR = 1.03, 95%CI = 0.68-1.04), HTN (HR = 0.87, 95%CI = 0.21-3.85), type II DM (HR = 0.69, 95%CI = 0.19-2.52), NLR (HR = 1.03, 95%CI = 0.92-1.15), CEA (HR = 1.00, 95%CI = 1.00-1.01), CA 19-9 (HR = 1.02, 95%CI = 0.99-1.04), and tumor characteristics. Notably, in stage III colon cancer, male patients (HR = 6.16, 95%CI = 1.24-30.10) with LVI (HR = 4.62, 95%CI = 1.17-18.33) had a higher risk of extranodal TDs.

Table 1 Baseline characteristics of N1c group and non-N1c group, *n* (%)

Item	N1c	Non-N1c	*P* value
Sample size (*n*)	19	136	
Gender			0.08
Male	13 (68.4)	64 (47.1)	
Female	6 (31.6)	72 (52.9)	
Mean age (yr)	66.79	65.17	0.09
BMI (kg/m^2)	22.77	23.41	0.15
Habit of smoking	8 (42.1)	46 (33.8)	0.48
Habit of alcoholic drinking	4 (21.1)	13 (9.6)	0.13
Hypertension	6 (31.6)	46 (33.8)	0.85
Diabetes mellitus	4 (21.1)	29 (21.3)	0.98
Neutrophil to Lymphocyte ratio	4.33	4.62	0.42
CEA (mg/dL)	12.83	18.23	0.62
CA 19-9 (mg/dL)	23.61	29.14	0.20
Tumor characteristics			
Location			0.27
Right colon	6 (9)	61 (91)	
Left colon	13 (14.8)	75 (85.2)	
T stage			0.90
T1	1 (5.3)	5 (3.7)	
T2	1 (5.3)	12 (8.8)	
T3	16 (84.2)	110 (80.9)	
T4a	0 (0)	4 (2.9)	
T4b	1 (5.3)	5 (3.7)	
Mean tumor size (cm)	4.24	4.8	0.98
Tumor type			0.26
Polypoid	8 (42.1)	76 (55.9)	
Ulcerative	11 (57.9)	60 (44.1)	
Tumor grade			0.62
Well	0	5 (100)	
Moderate	16 (13.3)	104 (86.7)	
Poor	3 (10.0)	27 (90.0)	
EGFR	17 (89.5)	117 (86.0)	0.68
Lymphovascular invasion	3 (15.8)	53 (39.0)	0.049

95%CI: 95% confidence interval; BMI: Body mass index; NLR: Neutrophil to lymphocyte ratio; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

Overall survival

The overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively (P = 0.027, Figure 3A), indicating that patients without LVI had higher overall survival than those with LVI. Furthermore, we divided the patients into N1c and non-N1c groups for subgroup analysis in regards to the impact of LVI on overall survival. In the non-N1c group, the overall survival rates of the patients with and without LVI were 6.91 years and 8.56 years, respectively (P = 0.13, Figure 3B), whereas the overall survival rates of the individuals with and without LVI in the N1c group were 4.42 years and 7.73 years, respectively (P = 0.01, Figure 3C).

Table 2 N1c group *vs* non-N1c group by multivariable cox regression

Item	Multivariable logistic regression		
	Exp (B)	95%CI	*P* value
Gender			
Female	Reference		
Male	4.62	(1.17-18.33)	0.030
Age	1.03	(0.99-1.08)	0.190
BMI	0.84	(0.68-1.04)	0.110
Habit of smoking	0.92	(0.27-3.07)	0.890
Habit of alcoholic drinking	1.41	(0.29-6.81)	0.670
Hypertension	0.87	(0.21-3.85)	0.850
Diabetes mellitus	0.69	(0.19-2.52)	0.570
Neutrophil to Lymphocyte ratio	1.03	(0.92-1.15)	0.660
CEA	1.00	(1.00-1.01)	0.770
CA 19-9	1.02	(0.99-1.04)	0.200
Tumor characteristics			
Location			
Right colon	Reference		
Left colon	1.67	(0.45-6.21)	0.440
T stage			
Tumor size	0.99	(0.96-1.02)	0.550
Tumor type			
Polypoid	Reference		
Ulcerative	1.75	(0.62-4.92)	0.290
Tumor grade	0.51	(0.12-2.21)	0.370
EGFR	1.05	(0.20-5.48)	0.950
Lymphovascular invasion	6.16	(1.24-30.10)	0.027

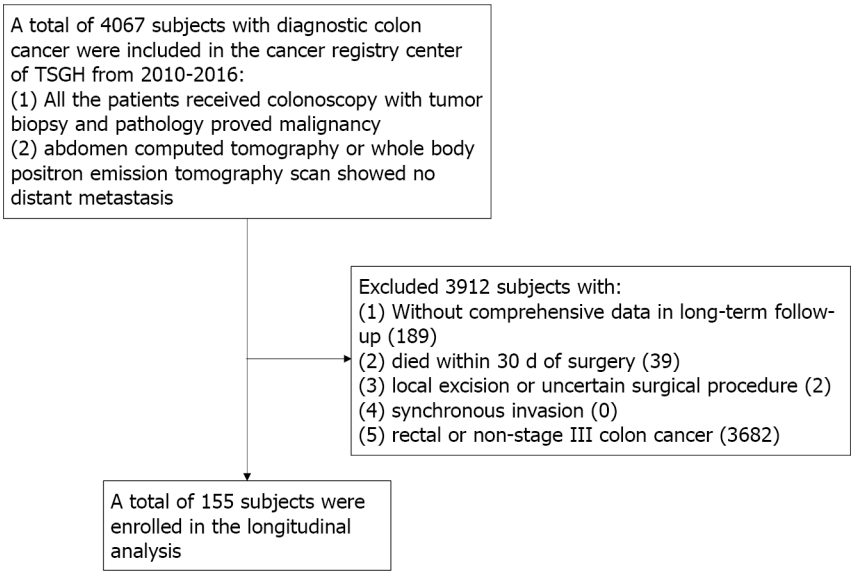
95%CI: 95% confidence interval; BMI: Body mass index; NLR: Neutrophil to lymphocyte ratio; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

Disease-free survival

The disease-free survival rates of patients with and without LVI were 5.92 years and 8.16 years, respectively (P = 0.005, Figure 4A), indicating that patients without LVI had higher disease-free survival than those with LVI. As before, subgroup analysis of LVI was performed by patient grouping into N1c and non-N1c groups. The disease-free survival rates of the patients in the non-N1c group with and without LVI were 5.98 years and 8.18 years, respectively (P = 0.013, Figure 4B). However, in the N1c group, the disease-free survival rates were 4.42 years and 7.56 years, respectively, for patients with and without LVI (P = 0.097, Figure 4C).

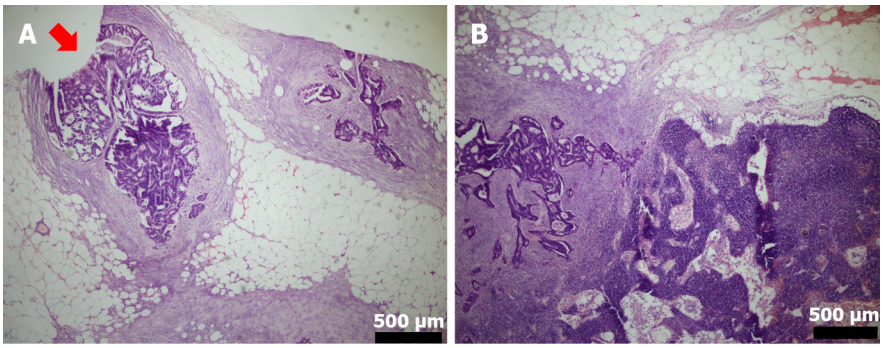
DISCUSSION

Patients with stage III colon cancer are contemplated to have a clinically significant hazard of distant metastasis after surgical resection. However, individuals with stage III colon cancer have a spectrum of risk of progressive disease. Beside tumor stage, the NCCN identifies LVI, TDs, and perineural invasion as histopathological characters related with patient survival[15]. In addition, TDs have been comprehensively studied in colon cancer, with most studies representing that they are an essential prognostic variable[15]. Some studies have even stated that TDs and tumor budding are the only histological variables that individually predict tumor recurrence in stage III colon cancer and should be comprised



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Figure 1 Description of the study flowchart.



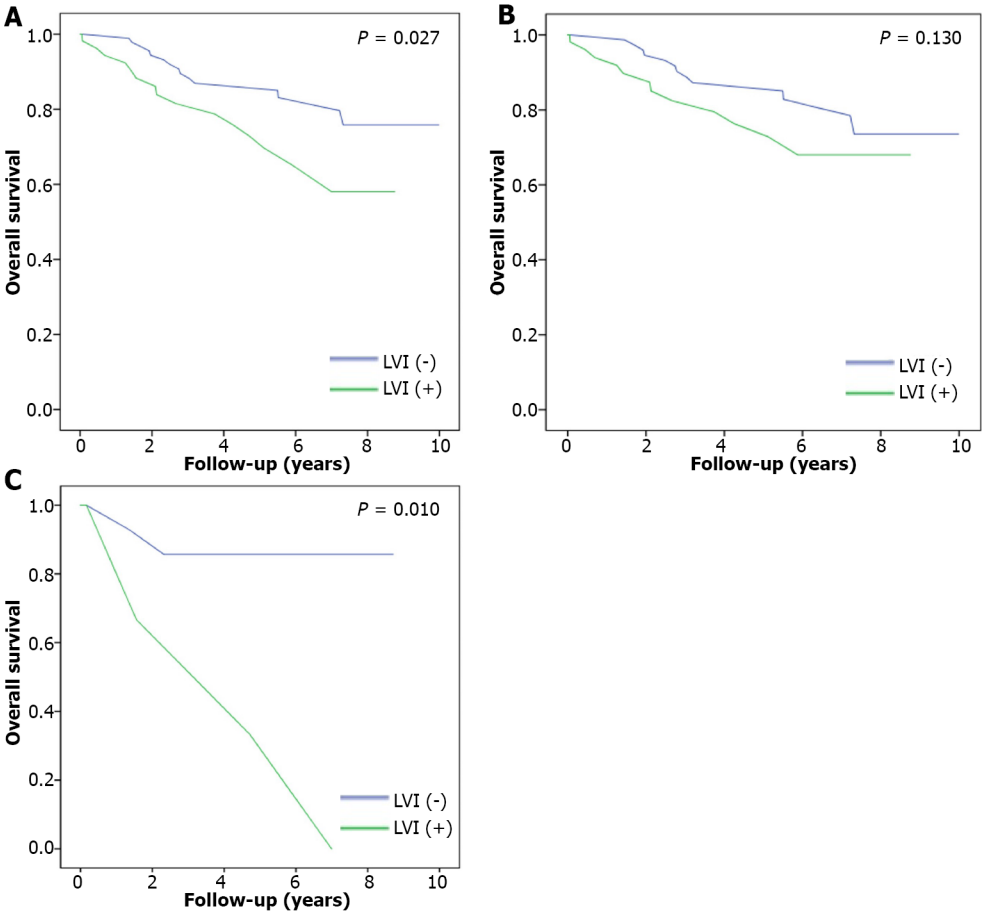
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Figure 2 Hematoxylin-eosin staining. A: The hematoxylin-eosin (HE) staining of N1c; B: The HE staining of non-N1c.

as part of a regular comprehensive pathological risk appraisal. TDs are defined as a discrete focus of tumor within the lymph node drainage area of the primary carcinoma with no distinguishable lymph node[15].

TDs are defeined as extramural focal aggregates of cancer cells located in the peritumoral fatty tissue (either mesocolon or mesorectum), which have no continuity with the main tumor mass and are not associated with a lymph node[16]. However, there is still a debate about what TDs really are, as they usually share different morphologies that make their origin unclear[16]. Some studies believe that TDs simply represent a stage of the LVI and/or perineural invasion process during which malignant cells begin to proliferate, giving rise to distinct nodules of cancer[16] that have to be distinguished from the involved lymph nodes. Other studies believe that TDs either represent a sporadic tumor spread, a totally replaced lymph node (LN), venous invasion with extravascular extension, and/or less commonly, a small vessel or perineural invasion[17]. TDs are generally present in about 4.5%-45.0% of CRC patients[16], while their incidence looks to be greater in advanced and/or metastatic tumors[16]. Jin *et al*[17] demonstrated that about 10% of CRCs have TDs, and 2.5% of colon cancers and 3.3% of rectal cancers have TDs without positive LNs.

Interobserver variability exists among pathologists in interpreting TDs[17]. It is clear that the determination of TD remains subjective, and no single criterion or group of criteria are comprehensively used or agreed upon. However, knowledge of the potential challenges and possible solutions may help reduce interobserver variability. In our study, we used the multivariable Cox regression model to analyze characteristics, including age, sex, comorbidities, tumor location, tumor staging, and tumor markers, of the CRC patients. We showed that LVI could predict CRC patients with N1c component, and this could allow pathologists to pay more attention to this subgroup of patients.



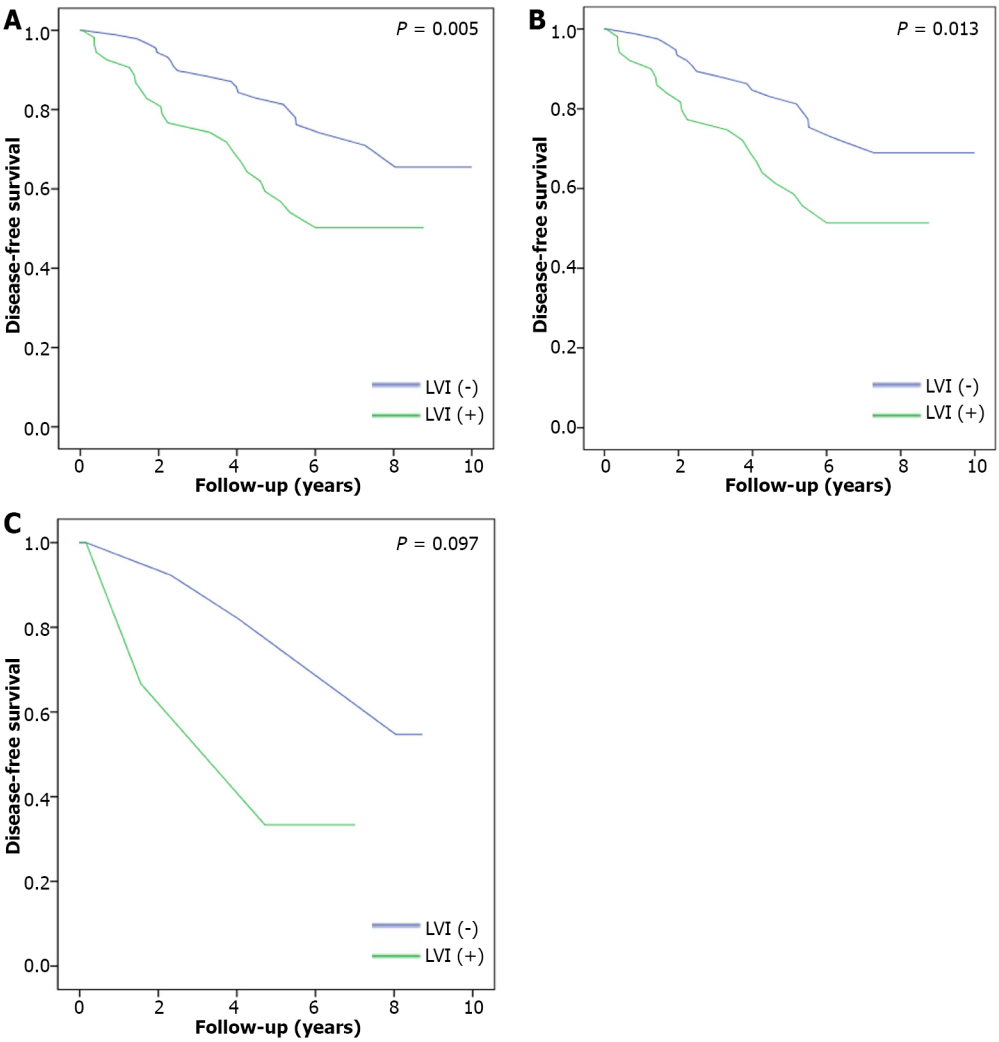
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Figure 3 Overall survival. A: The overall survival of the patient with/without lymphovascular invasion (LVI); B: The overall survival of the non-N1c patient with/without LVI; C: The overall survival of the N1c patient with/without LVI.

LVI positivity, characterized by the extension of tumor cells into lymphatic and/or blood vessels, has long been recognized as a probable indicator of lymph node metastasis, prognostic indicator, and predictor of patient outcomes. Many studies have investigated the presence of LVI in CRC and have determined it to be a strong stage-independent prognostic marker[18]. Patients with LVI usually have a higher chance of disease progression and poorer prognosis[18]. On the other hand, in recent years, TDs have become a hotspot in colon cancer study. In the seventh and eighth editions of the AJCC staging system, TDs were included in the nodal staging[19]. TD patients without regional lymph node metastasis were correlated with other high-risk characters, because there was more LV and perineural invasion in this group. This finding correlates with histopathologic results in other studies because TDs were revealed to be of perineural origin in 77% of cases, intravascular origin in 83% of cases, and a combined perineural, perivascular, and intravascular origin in 40%[20].

In a recent systematic review and meta-analysis of stage I-IV CRC, TDs were always related with worse overall survival and disease-free survival[20]. One study stated that the survival curves of all patients in stages I-III with TDs were more similar to the survival curves of the stage IV than stage III patients, and patients with TDs in stages I-III showed similar mortality rates as stage IV patients[21]. Another up-to-date study has indicated that the presence of TD in individuals with stage III colon cancer is related with a 2.2-fold increased risk of developing disease recurrence[22]. In our study, the results reported that LVI could predict TDs in patients with stage III colon cancer. The subgroup of N1c stage III colon cancer patients with LVI showed poor prognosis regarding overall survival, while the non-N1c subgroup patients showed no significant difference.

Despite showing that the predicted risk factor of LVI makes the prognostic significance of TDs in stage III colon cancer patients more promising, there are still some limitations to the study. This is a retrospective study of observational data using a small sample of patients, the prevalence of N1c in colon cancer could as low as 1.59% in the previous study[23], which might result in statistical bias with inconsistent results between overall and disease-free survival. Further prospective studies with more patients involved might address our result more promising. There might also be systematic differences in the pathological evaluation of the surgical specimens, which may have biased the outcomes.



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Figure 4 Disease-free survival. A: The disease-free survival of the patients with/without lymphovascular invasion (LVI); B: The disease-free survival of the non-N1c patient with/without LVI; C: The disease-free survival of the N1c patient with/without LVI.

CONCLUSION

Patients with stage III colon cancer with LVI might be more likely to have TDs. Interobserver variability among pathologists and the multidisciplinary committee might at times influence consistent interpretation and reporting, and the frequent association between co-occurrence of TDs and LVI may postulate extra insight into the nature and derivation of TDs. Pathologist should not miss these subgroups of N1c patients, because TDs in combination with LVI could predict poor patient outcomes. Greater attention must be paid to the subject of TD positivity and prompt suitable risk stratification by considering a more robust therapeutic approach and closer survivorship planning for this subgroup of high-risk stage III colon cancer patients, who might be undertreated and require adjustment of adjuvant chemotherapy regimens. Amendment in the delivery of proper care to these patients may increase survival and should be an object of future quality ambition.

ARTICLE HIGHLIGHTS

Research background

In the American Joint Committee on Cancer (AJCC) TNM staging system, tumor deposit (TD) was classified as pN1c in stage III colon cancer patients without lymph node metastasis, but extranodal deposits are a distinct form of metastatic disease in patients with colon cancer in some studies.

Research motivation

To conduct a retrospective study to investigate risk factors for extranodal TDs in stage III colon cancer.

Research objectives
We used SPSS Statistics software. Student's *t*-test and the chi-square test were utilized to investigate quantitative variables and qualitative variables. Multivariate Cox regression analysis was performed to investigate the association between the covariates and extranodal TDs. The Kaplan-Meier method was utilized to analyze the overall survival and disease-free survival rates.

Research methods
We selected 155 patients diagnosed with stage III colon cancer from the database of the Cancer Registry of the Tri-Service General Hospital retrospectively. The patients were categorized into the groups with/without N1c. Multivariate Cox regression analysis and Kaplan-Meier method were done. The primary outcomes investigate the association between the covariates and extranodal TDs, and prognostic significance of the covariates regarding the survival.

Research results
Patients with lymphovascular invasion (LVI) had a higher risk of TDs. Overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively. The N1c patients without LVI had higher overall survival than those who with LVI.

Research conclusions
Stage III colon cancer patients with TDs and LVI could have poor prognosis and outcome.

Research perspectives
Greater attention must be paid to the issue of TD. Amendment in the delivery of proper care to these patients may increase survival and should be a target of future quality ambition.

FOOTNOTES

Author contributions: Jhuang YH contributed to conceptualization, data curation, formal analysis, investigation, methodology, validation, and writing original draft; Chou YC contributed to methodology, software and supervision; Lin YC, Hu JM, and Pu TW contributed to data collection; Chen CY contributed to supervision, validation, review and editing.

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ORIGINAL ARTICLE

Retrospective Study

Outcomes of ABO-incompatible liver transplantation in end-stage liver disease patients co-infected with hepatitis B and human immunodeficiency virus

Jian-Xin Tang, Kang-Jun Zhang, Tai-Shi Fang, Rui-Hui Weng, Zi-Ming Liang, Xu Yan, Xin Jin, Lin-Jie Xie, Xin-Chen Zeng, Dong Zhao

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Abstract

BACKGROUND

Human immunodeficiency virus (HIV)-positive patients coinfected with hepatitis B virus (HBV) are eligible for liver transplantation (LT) in Africa and Southeast Asia, particularly China. However, the outcome of HIV-HBV coinfected patients referred for ABO-incompatible LT (ABOi-LT) is unknown.

AIM

To clarify the outcome of ABOi-LT for HIV-HBV coinfected patients with end-stage liver disease (ESLD).

METHODS

We report on two Chinese HIV-HBV coinfected patients with ESLD who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. The pretransplantation HIV viral load was undetectable, with no active opportunistic infections. Induction therapy consisted of two sessions of plasmapheresis and a single dose of

rituximab in two split doses, followed by an intraoperative regimen of intravenous immunoglobulin, methylprednisolone, and basiliximab. Post-transplant maintenance immunosuppressive agents consisted of tacrolimus and mycophenolate mofetil, and prednisone.

RESULTS

At the intermediate-term follow-up, patients showed undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. A liver allograft biopsy showed no evidence of acute cellular rejection. Both patients survived at 36-42 mo of follow-up.

CONCLUSION

This is the first report of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, suggesting that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

Key Words: ABO incompatibility liver transplantation; Human immunodeficiency virus; Hepatitis B virus; End-stage liver disease; Immunosuppression

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Core Tip: The outcome of human immunodeficiency virus (HIV)-hepatitis B virus (HBV) coinfected patients referred for ABO-incompatible liver transplantation (LT) (ABOi-LT) is unknown. We report on two Chinese HIV-HBV coinfected patients with end-stage liver disease (ESLD) who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. At intermediate-term follow-up, patients showed undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. Both patients survived at 36-42 mo of follow-up. This is the first report of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, suggesting that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

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INTRODUCTION

The recent introduction of highly active antiretroviral therapy (HAART) has dramatically changed the natural history of human immunodeficiency virus (HIV) infection[1]. Both the mortality rate and the incidence of acquired immune deficiency syndrome (AIDS) due to HIV infection have decreased with effective suppression of viral replication and prophylaxis against opportunistic infections[2]. However, HIV-infected patients are frequently coinfected with hepatitis B virus (HBV) since both viruses share similar modes of transmission, resulting in an increased risk of developing chronic liver disease[3,4]. After the dramatic improvement in the survival of HIV-infected patients with HAART, hepatitis cirrhosis and its complications have replaced opportunistic infections as the leading cause of mortality in the HIV-HBV coinfected patient[5]. In addition, almost all of the antiretroviral agents are metabolized in the liver. Patients with hepatic metabolic impairment cannot use these agents, accounting for the increased mortality associated with AIDS[6]. Accordingly, end-stage liver disease (ESLD) accounts for up to 50% of deaths in HIV-infected patients[7,8].

It has long been thought that HIV is a contraindication to liver transplantation (LT) in the pre-HAART era since immunosuppression can reportedly aggravate HIV infection and complications[9]. Case reports have shown nearly 25% AIDS-related mortality in HIV patients 6 mo after transplantation [10,11]. However, an increasing body of evidence suggests comparable survival rates between HIV-positive and HIV-negative recipients after LT in the HAART era[12-14]. These results suggest that HIV infection should not be a contraindication to LT, provided the underlying HIV disease is under control. Recent studies have shown that common indicators of controlled HIV disease-infected patient pretransplantation include an HIV viral load < 200 copies/mm^3, a CD4(+) T cell count greater than 200 cells/μL, and the absence of active opportunistic infections for at least 6 mo[15].

ABO-incompatible LT (ABOi-LT) is considered to be a high-risk procedure, compared to ABO-compatible LT, associated with a higher rate of antibody-mediated rejection, biliary complications, hepatic artery thrombosis, and mortality[16]. Hence, ABO-incompatible liver grafts have been used as a rescue option. Advances in the treatment strategies for ABOi-LT[17] include plasmapheresis, intravenous immunoglobulin (IVIG), splenectomy, rituximab, antilymphocyte antibodies, and immunosuppressant medications have improved the post-LT outcomes. The past decade has witnessed an increase in ABOi-LT procedures with increasing success. No significant difference between rejection and allograft survival at 1, 3, and 5 years after transplantation was found in a United Network of Organ Sharing Database analysis between 1990 and 2010 that compared ABOi liver transplants with ABO-compatible transplants[18].

Notably, the China Liver Transplant Registry does not prohibit HIV patients from receiving organs. With the introduction and effectiveness of HAART therapy, the outcomes of HIV-positive recipients with ESLD are reportedly similar to HIV-negative recipients after LT[19]. However, the incompatibility between the ABO blood group and living organ donation severely limits the transplantation opportunities for this patient population[20]. Besides, LT in HIV patients using ABOi organs brings additional complexity and difficulty to this already intricate patient population. Therefore, assessing the practicability of ABOi-LT in HIV-positive recipients is essential. To our knowledge, ABOi-LT in an HIV-HBV coinfected recipient with ESLD has hitherto not been documented in the literature. Here, we report on two cases and review major clinical and research issues related to HIV-HBV coinfected patients treated with ABO-compatible LT. We present the following article in accordance with the AME Case Series reporting checklist.

MATERIALS AND METHODS

From January 2019 to December 2021, 7 patients with HIV infection underwent LT in our LT center, including 2 patients with ABOi-LT. These 2 patients underwent ABOi-LT between April 2019 and December 2019, and their clinical data were extracted from our database. Both patients received HAART and anti-HBV therapy before transplantation and presented undetectable HIV RNA and HBV DNA levels, while the CD4(+) T cell count of one patient was less than 100 cells/μL. The surgical technique of ABOi-LT was a modified piggyback technique with triangulation of the hepatic veins. The vena cava anastomosis was completed with three separate continuous sutures, first completing the right side of the triangle. Subsequently, we released the vena cava blood flow to reduce the cold ischemia time. Next, we successively performed portal vein and hepatic artery vascular anastomosis. Bile duct reconstruction was performed by end-to-end anastomosis (continuous for the posterior wall and interrupted for the anterior wall). The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This retrospective study was a single-center consecutive case series analysis approved by the Ethics Committee of the Third People's Hospital of Shenzhen (No. 2022-038-02). Written informed consent was obtained from all participants.

To the best of our knowledge, no cases of ABO incompatibility involving HIV-HBV coinfection recipients have been reported in the literature. Little information is available on HIV-HBV coinfected patients that undergo LT and methods to improve survival and reduce complications. We systematically searched for all patients diagnosed with HIV who underwent LT from 1995 to 2022. Search terms were (HIV or human immunodeficiency virus) AND (HBV or hepatitis B virus) AND (liver or hepatic) AND (transplantation or transplant) in Embase, MEDLINE, and PubMed. When data were missing, we contacted the study authors for additional information.

RESULTS

Clinical data of patients

Case 1: A 61-year-old HIV-positive Chinese man with grade IV hepatic encephalopathy and hepatorenal syndrome secondary to decompensated HBV cirrhosis was referred for LT. He was considered a good candidate for LT because he had undetectable HIV RNA levels for at least 5 years, the CD4(+) T cell count was 42 cell/μL, and he received lamivudine and efavirenz as part of his Reverse Transcriptase inhibitor-based HAART therapy regimen for approximately 6 years. The model for ESLD (MELD) score was 40. However, given the absence of a suitable ABO-compatible liver donor and the patient's critical condition requiring urgent LT, a man with blood type A+ was used as the donor (O+ recipient, A+ donor). The recipient's latest anti-A titers were 1:128 for immunoglobulin G (IgG) and 1:32 for immunoglobulin M (IgM) before transplantation.

The decision was made to proceed with LT on April 4, 2019. Induction therapy consisted of two sessions of plasmapheresis, and rituximab 375 mg/m^2 in split doses was administered intravenously

before surgery. IVIG 400 mg/kg, rituximab 375 mg/m^2 in split doses, and methylprednisolone 0.5 g were administered intravenously during the operation; and basiliximab 20 mg was administered intravenously prior to the release of circulation in the graft.

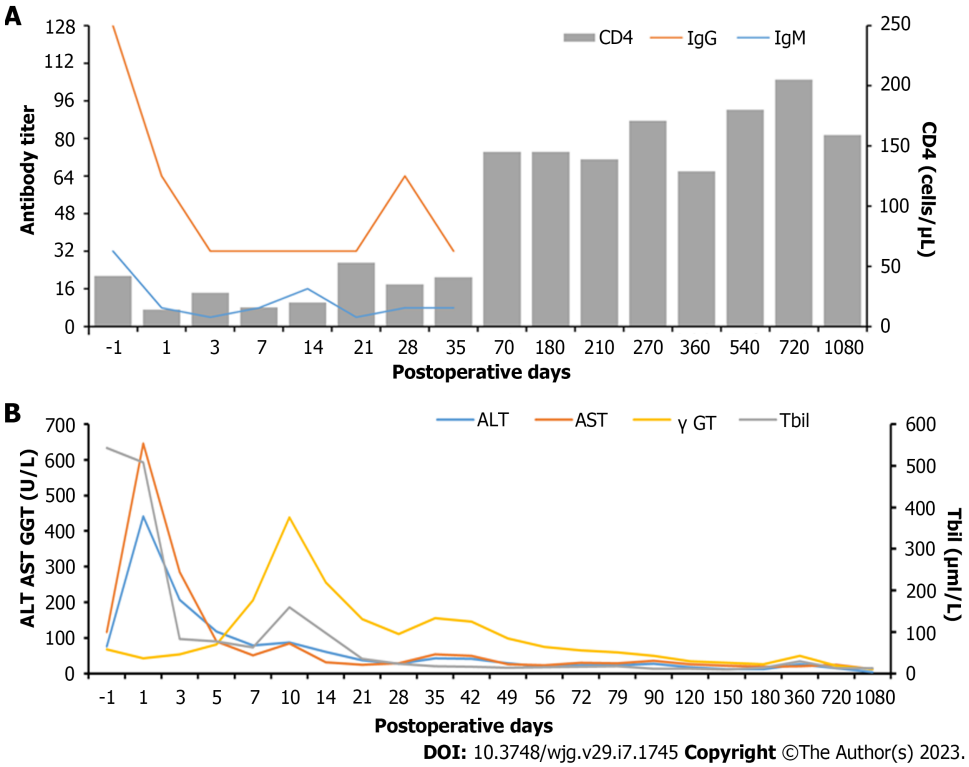
Following transplantation, the patient was started on methylprednisolone for 7 d and switched to prednisone 48 mg daily and tapered to 8 mg daily over 4 wk. Maintenance immunosuppressive agents included tacrolimus and mycophenolate mofetil. Initially, 1 mg tacrolimus twice daily was started on postoperative day (POD) 1 and titrated to achieve a trough of 12 to 15 ng/mL during the first 2 postoperative weeks. The tacrolimus dose was adjusted to maintain a concentration of 8 to 12 ng/mL from 2 wk to 1 mo after transplantation, 6 to 8 ng/mL within 1 to 6 mo after transplantation, and then 4 to 6 ng/mL for long-term follow-up in our institution. Mycophenolate mofetil was started on POD4 and adjusted reasonably according to the postoperative liver function, infection index, white blood cell count, and drug concentration. IVIG 400 mg/kg was given daily for 7 d, then every other day for two more sessions. In addition, the second dose of basiliximab 20 mg was given on POD4. HAART therapy consisting of dolutegravir and lamivudine was restarted on POD1. Post-transplant anti-HBV therapy included tenofovir alafenamide and monthly infusions of high-dose hepatitis B immune globulin (HBIG). Given the presence of HIV-associated immunodeficiency and antirejection medication use, infection prophylaxis is critical. The patient was treated with antibiotics, ganciclovir, and antifungal combination therapy.

Due to a high preoperative anti-A IgM/IgG titer, the anti-A IgM/IgG titer, CD4(+) T lymphocyte count, postoperative rejection, and the risk of opportunistic infection after LT were dynamically monitored. From POD1, ABO blood group antibody titers and T lymphocyte subsets were detected in the blood collected from the patient every other day. Postoperative monitoring showed that anti-A IgG/IgM decreased from 1:128/1:32 to 1:32/1:16 at 2 wk postoperatively (Figure 1A). There were no problems with postoperative infection and HIV management; the patient received post-transplant HAART and anti-HBV therapy with CD4(+) T cell counts ranging from 60 to 148 cells/μL with undetectable HIV RNA and HBV DNA levels. Allograft ultrasound showed normal blood vessels and biliary tract. Serum transaminase peaked at 458 U/L on POD1, then began to trend down. However, graft function worsened on POD14. Mild elevation of the hepatic enzymes [total bilirubin (Tbil): 97 μmol/L; γ-glutamyltransferase: 255 U/L; aspartate aminotransferase: 39 U/L; alanine aminotransferase (ALT): 61 U/L] was observed (Figure 1B). The IgG and IgM titers remained stable (1:32 and 1:16, respectively). The follow-up liver allograft biopsy showed mild acute cellular rejection, with a Banff rejection activity index score of 4 (Figure 2A). After the patient received intravenous pulse steroid therapy (80 mg of methylprednisolone for 3 d, then tapered), liver enzymes decreased and subsequently remained in the normal range. The patient was discharged on POD37 without any infection and exhibited normal liver function. On subsequent clinical follow-up, normal hepatic enzymes were maintained. His latest CD4(+) T cell count was 159 cells/μL on June 6, 2022. A follow-up liver biopsy 1 year after transplantation revealed no evidence of graft rejection. More than 3 years after LT, the patient and graft function remained stable.

Case 2: A 46-year-old HIV-HBV coinfected Chinese man with acute-on-chronic liver failure was referred for LT. The preoperative Tbil was 320 μmol/L, and the international normalized ratio was 9.85. A multidisciplinary team discussion concluded that he was considered a good candidate for emergency LT with a MELD score of 40 and received a HAART therapy regimen for approximately 3 years with undetectable HIV RNA levels and a CD4(+) T cell count of 120 cells/μL. However, in the absence of suitable ABO-compatible liver donors, a man with blood type A+ was used as the donor (O+ recipient, A+ donor). The recipient's most recent anti-A titers for IgG and IgM were both 1:32 before transplantation. On November 19, 2019, the patient underwent ABOi-LT in our LT center. The induction treatment and maintenance immunosuppression scheme is shown in patient 1. HAART therapy was restarted on POD1 without changes. Given the immunodeficiency status of the patient treated with antirejection medication, the patient was treated with antibiotics, ganciclovir, and an antifungal combination therapy for infection prophylaxis.

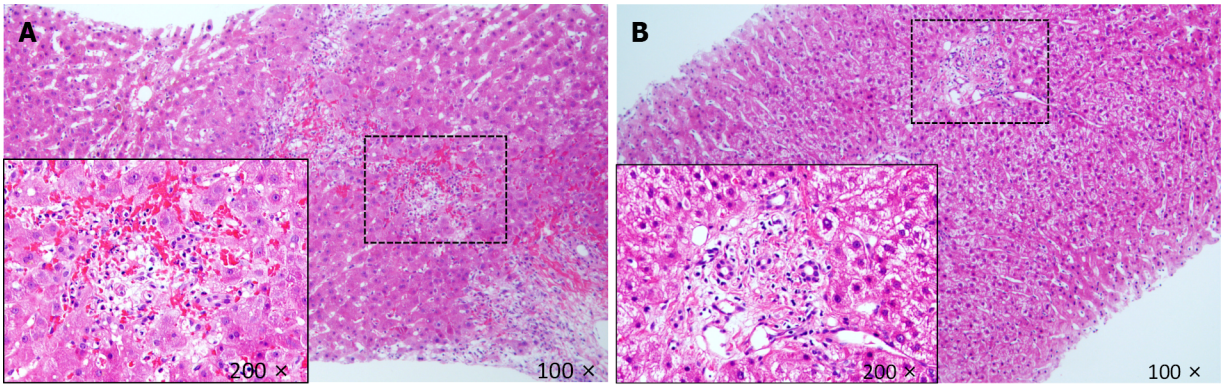
The baseline anti-A IgG/IgM titers were both 1:32. Although the baseline anti-A titer was high and the CD4(+) T cell count was less than 200 cells/μL, an emergency ABOi-LT was successfully performed. The anti-A IgG/IgM titer, CD4(+) T cell count, postoperative rejection, and the risk of opportunistic infection after LT were dynamically monitored. Postoperatively the anti-A IgG/IgM titers decreased from 1:32/1:32 to 1:16/1:4 at 2 wk. Postoperative infection was not observed, and HAART and anti-HBV therapy were continued; the post-transplant CD4(+) T cell counts ranged from 62 to 494 cells/μL with undetectable HIV RNA and HBV DNA levels. The patient was finally discharged on POD63 with normal liver function, IgG and IgM titers of 1:8 and 1:4, respectively, and the absence of any complications (Figure 3A).

Deteriorating graft function was observed three months after LT, with relatively stable liver function (Figure 3B). The blood drug concentration of tacrolimus was 3 ng/mL, and the CD4(+) T-cell count was 390 cells/μL. A follow-up liver biopsy at 6 mo after transplantation revealed no evidence of graft rejection. Twenty-five months after LT, he was hospitalized due to abnormal hepatic enzymes and underwent a liver biopsy. The pathology report suggested chronic cholangitis with bile duct sclerosis and the possibility of early chronic rejection (Figure 2B). After the adjustment of the immunosup-



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Figure 1 Dynamic changes in immunological indicators and liver function in case 1. A: Trends of anti-A immunoglobulin M (IgM)/immunoglobulin G (IgG) titers and CD4(+) T cell counts over time; B: Trends of liver enzymes over time after liver transplantation. γGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Tbil: Total bilirubin.



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Figure 2 Pathological results of liver allograft biopsy. A: Patient 1. The pathology results of liver allograft biopsy showed mild portal inflammation, mild venous endothelial inflammation, mild small bile duct inflammation and capillary bile duct cholestasis suggesting mild acute cellular rejection (Banff rejection activity index score, 4); B: Patient 2. The pathology results suggested chronic interlobular cholangitis with bile duct sclerosis and the possibility of early chronic rejection.

pressive drug regimen, the patient's liver function gradually stabilized. During the subsequent follow-up, the hepatic enzymes remained within the normal range with a CD4(+) T cell count of 467 cells/μL on February 7, 2022. Nearly 36 mo after LT, the patient and graft function remained stable.

Literature review

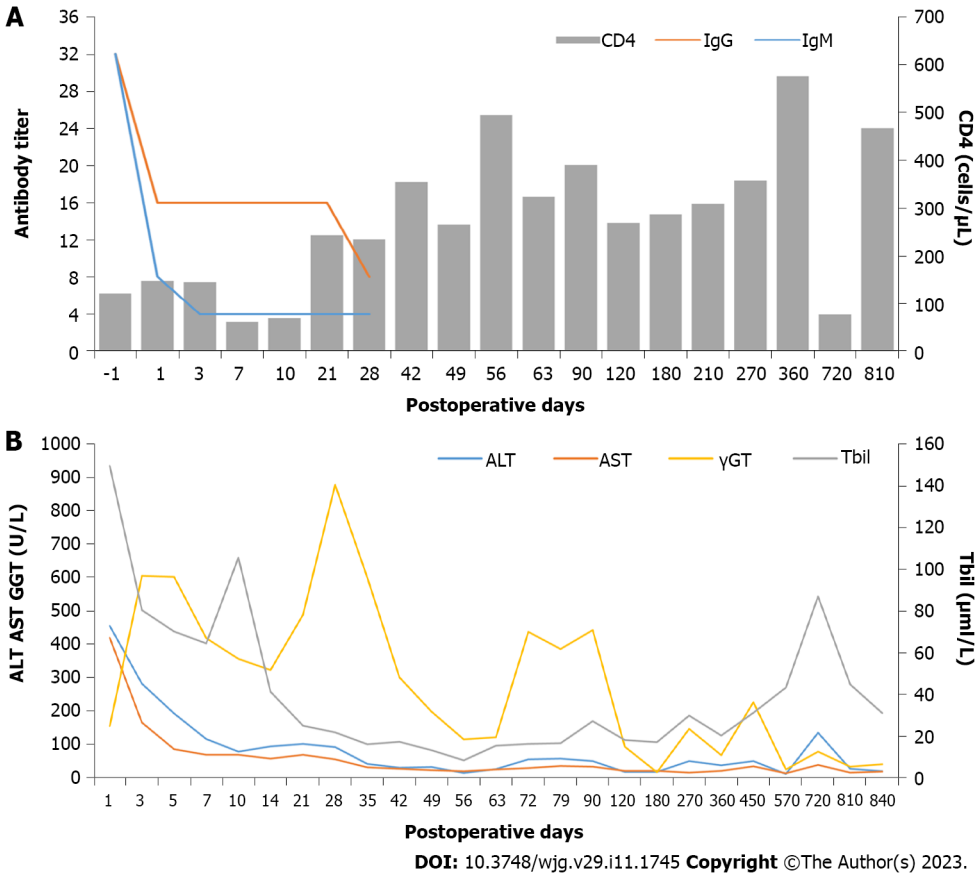
To date, there have been no reports of successful ABOi-LT in HIV-HBV coinfected patients. To understand the prognosis and risk of HIV complications in patients with simultaneous HBV infection after LT, we reviewed the literature for LT in HBV-HIV coinfected patients[12-15,21-27]. Eleven studies were screened, reporting the characteristics of 69 patients with HIV-HBV coinfection that underwent LT from 1995 to 2022 (Table 1). The etiology of liver disease was HBV-related cirrhosis (n = 62) and fulminant liver failure due to HBV (n = 7).

Table 1 Summary of outcomes pre- and post-liver transplantation in human immunodeficiency virus-hepatitis B virus coinfected patients

Ref.	Patients, *n*	Study period in yr	Pre-LT				Post-LT					
			Liver disease	CD4(+) T cell count as cells/μL	HIV-RNA as copies/mm^3	HBV-DNA as IU/mL	Median follow-up in mo	Latest CD4(+) T cell count as cells/μL	Latest HIV-RNA as copies/mm^3	Latest HBV-DNA as IU/mL	Rejections, *n*	Survival after LT in mo
Tateo *et al*[12]	13	1999-2007	HBV	173 (118-615)[1]	100% < 40	100% < 12	32 ± 5.2[2]	281 (10-810)[1]	100% < 40	100% < 12	2	100% 1 yr, 100% 3 yr, 100% 5 yr
Schreibman *et al*[27]	6	1999-2006	5 HBV, 1 ALF	100% > 100	100% < 200	66.7% < 12	60 (2-64)[1]	83.3% > 100	100% < 100	83.3% ND	1	66.7% 1 yr, 66.7% 3 yr, 66.7% 5 yr
Norris *et al*[25]	5	1995-2003	4 HBV, 1 ALF	187 (124-293)[1]	20% < 50	100% < 12	15 (6-65)[1]	467 (241-754)[1]	100% < 50	100% < 12	1	100% 1 yr, 100% 3 yr
Coffin *et al*[15]	22	2001-2007	21 HBV, 1 ALF	317 (38–1070)[1]	100% < 40	45.4% ND	42 (0.6-84)[1]	289 (48–744)[1]	NA	68% ND	5	85% 1 yr, 85% 3 yr
Vernadakis *et al*[1]	2	1996-2009	1 HBV, 1 ALF	219, 403	50% < 50	NA	3, 34	NA	NA	NA	0	3, 34
Neff *et al*[22]	4	1997-2001	2 HBV, 2 ALF	75% > 100	50% < 50	ND	21 (5-36)[1]	100% > 100	100% < 50	ND	3	100% 1 yr, 100% 3 yr
Anadol *et al*[23]	10	1997-2011	HBV	100% > 100	80% < 50	ND	NA	NA	ND	ND	1	80% 1 yr, 80% 3 yr, 80% 5 yr
Radecke *et al*[24]	1	1998-2001	HBV	196	380	NA	3	> 100	NA	NA	1	3
Schliefer *et al*[26]	1	1997-1999	ALF	477	< 80	NA	27	> 100	< 80	NA	0	27 (Alive)
Roland *et al*[21]	1	NA	HBV	439	ND	ND	20	305 to 700	ND	ND	0	20 (Alive)
Terrault *et al*[1]	4	2000-2002	HBV	175 (104-439)	100% < 75	ND	18, 25, 42, 48	315 (125-505)	ND	ND	0	100% 1 yr, 100% 3 yr

[1]Median (range);
[2]mean ± SD.
ALF: Acute liver failure; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; NA: Not available; ND: Not detectable; LT: Liver transplantation.

Fifty-nine patients had HIV infection under control, with undetectable or low HIV viral loads and no previous AIDS events or opportunistic infections upon LT waiting list registration. At the time of transplantation, all but one patient had CD4(+) T cell counts above ≥ 100 cells/μL[22]. Polymerase chain reaction showed that 17.4% of patients (12/69) had detectable HBV DNA prior to transplant (among these, 10 patients received adefovir and/or entecavir therapy, and 2 patients received lamivudine



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Figure 3 Dynamic changes in immunological indicators and liver function of case 2. A: Trends of anti-A immunoglobulin M (IgM)/immunoglobulin G (IgG) titers and CD4(+) T cell counts over time; B: Trends of liver enzymes over time after liver transplantation. γGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Tbil: Total bilirubin.

combined with tenofovir therapy).

All patients received HAART following LT and plasma HIV-RNA remained low to undetectable in all patients (one case presented with a viral load of 76 copies/mL) during follow-up. No viral breakthrough was observed. CD4(+) T cell counts were maintained at more than ≥ 100 cells/μL. All patients received dual immunoprophylaxis with hepatitis B immunoglobulin and anti-HBV medications. Eight patients tested positive for HBV DNA (among these, low-level HBV viremia was intermittently detected in 7 patients[15] but not associated with hepatitis B surface antigen detection or ALT elevation; for one patient[27] with a transiently positive HBV DNA, serum HBV DNA results were undetectable after tenofovir was added for antiviral therapy) (Table 1). 20.3% (14/69) of HIV-HBV coinfected patients were treated with high-dose prednisone or adjustment of immunosuppressive therapy after developing acute cellular rejection. However, one patient[23] died of hepatic artery thrombosis and graft failure due to rejection. The cumulative patient survival at one and three years in the HIV-HBV coinfected patients was 85.9% and 77.3%, respectively.

DISCUSSION

Current evidence suggests that in the era of HAART therapy, morbidity and mortality have declined in HIV-infected patients[28,29]. Mortality in this patient population is mainly attributed to comorbidities such as viral hepatitis infection, which is well-established to be associated with an increased prevalence of HIV-infected patients with ESLD. In recent years, studies have shown that the outcomes of HIV and non-HIV recipients were comparable, which has led to much controversy on transplantation in HIV patients[15]. Multiple centers have established protocols for HIV recipients, including an undetectable HIV RNA viral load and CD4(+) T cell counts greater than 200 cells/μL for sustained HAART therapy without other contraindications to LT. Overwhelming evidence[30-32] suggests promising outcomes for HAART-treated HIV-infected patients with maximally suppressed viral loads and no significant increase in opportunistic infections after LT.

Although LT is more common in selected HIV recipients, no studies have reported ABO incompatibility involving HIV-HBV coinfected recipients in the literature, while only 2 cases involving ABO-incompatible kidney transplantation have been reported in HIV recipients[33,34]. Our study provides the first documented cases of A to O incompatible LT in HIV-HBV coinfected recipients with ESLD. Ample evidence suggests that HIV-positive liver transplant recipients are prone to rejection due to immunosuppression or immune dysregulation from the virus itself[35-37]. Interestingly, the United Network of Organ Sharing Database[38] showed that the overall incidence of acute rejection in non-HIV recipients was 24.7% within 1 year after LT, which is similar to the incidence (20.3%) in our review of HIV-infected recipients. Regardless of the HIV infection status, acute rejection is common in ABOi transplantation, with an acute rejection rate of nearly 22%[18]. In our report, emergency ABOi-LT was successfully performed in HIV-HBV coinfected recipients that received induction therapy and adjunctive immunosuppressive regimens, including plasmapheresis, rituximab, basiliximab, methylprednisolone, and IVIG. Liver biopsy at mid-term follow-up did not show acute cellular rejection.

The two patients described in our study are the first cases of A to O brain-dead donor LT reported in the literature. The A-blood group is unique since it exhibits two phenotypes (A1 and A2) that harbor different immunogenicity. The A2 phenotype is characterized by reduced reactivity with anti-A isoagglutinin since it expresses fewer A epitopes on two of four possible core saccharide chains of the ABO antigen[39]. Besides, an increasing body of evidence[40,41] suggests that transplantation of A2 Liver grafts does not elevate anti-A titers after LT. Kluger *et al*[18] reported that blood group O recipients with A2 grafts exhibited no significant differences in rejection during the transplant hospitalization and at 12 mo postoperatively, with the same overall and graft survival rates as recipients with O grafts at 1, 5, and 10 years. The patient's preoperative anti-A IgG/IgM titers in case 1 were 1:128 and 1:32, respectively. No definite graft rejection was observed 3 years after LT because the donor's blood group was A2. Our center's protocol for A to O LT is based on baseline (pretransplantation) titer data. If the anti-IgG and the IgM titers are > 1:16, the preoperative management consists of two sessions of plasmapheresis, IVIG 400 mg/kg, and rituximab 375 mg/m^2 in split doses. In our article, both patients received this protocol. Postoperative anti-A titer monitoring in patient 1 showed that anti-A IgG/IgM decreased from 1:128/1:32 pre-LT to 1:32/1:16 at 2 wk. During the subsequent three years of clinical follow-up, the patient exhibited normal liver enzyme levels.

Preventing HBV recurrence and HIV progression has become a research hotspot in patients with HIV-HBV coinfection after LT. No consensus has been reached on the optimal anti-HBV therapy in patients with HIV-HBV coinfection. Anti-HIV drugs with anti-HBV activity include lamivudine, tenofovir, and emtricitabine. Drug therapy for HIV infection has important implications for preventing the recurrence of HBV infection. However, treating HBV infection in a coinfected patient with lamivudine or tenofovir alone can result in HIV resistance to these drugs, affecting anti-HIV treatment options in the future[42,43]. Accordingly, clinicians should be aware of the potential impact of nucleos/tide analogue selection on managing HIV-HBV coinfected recipients. Terrault *et al*[13] suggested that the combination therapy using nucleos(t)ide analogues and HBIG in the HIV-HBV coinfected recipient could effectively prevent post-transplantation HBV recurrence. In this report, the patient had excellent short outcomes after treatment with anti-HBV therapy, including tenofovir alafenamide and HBIG, and the HAART regimen consisting of dolutegravir and lamivudine. Subsequently, the two patients that underwent ABOi-LT with HIV were switched to albuvirtide and dolutegravir, well-recognized for their low hepatorenal toxicity and non-CYP450 enzyme inhibitors[44], reducing the impact of calcineurin inhibitor-type immunosuppressive drugs, which achieved intermediate-term excellent outcomes. Overall, it is essential to continuously monitor HIV RNA and HBV DNA levels and optimize anti-HIV and anti-HBV therapies to reduce postoperative complications and prolong survival in HBV-HIV coinfected recipients. Our HIV patients remained infection-free with good CD4(+) T cell count and stable liver function. Moreover, Albuvirtide and dolutegravir represent good options for HIV patients undergoing ABOi-LT.

Given that the number of HIV-infected patients with ESLD is expected to rise in the coming years, the same organ shortage issues that plague non-HIV patients will become increasingly severe for HIV patients[45]. To our knowledge, these two cases are the first reports of ABOi-LT in HIV-HBV coinfected patients with ESLD in the literature. In this study, the early course of ABOi-LT in HIV recipients was similar to that of ABO-compatible LT, without severe acute rejection. Desensitization regimens for ABOi-LT include rituximab, plasmapheresis, and IVIG. In previous studies[46,47] and our previous clinical practice, it has been observed that multiple doses of rituximab could increase the risk of infection, especially in immunodeficient HIV patients. Therefore, a sufficient dose and course of prophylactic antibiotics are crucial to prevent postoperative infection. Furthermore, the number of targeted B cells was significantly smaller in HIV transplant patients, and multiple doses of rituximab yielded no significant benefit for acute rejection or survival in transplant patients[48], suggesting that a single dose of rituximab may be sufficient. The desensitization protocol is usually initiated 2 to 3 d before transplantation. In these two cases, we administered a single dose of rituximab in two split doses, and two sessions of plasmapheresis were performed to reduce the anti-A titers with satisfactory results.

CONCLUSION

We successfully performed emergency ABOi-LT in HIV-HBV coinfected patients. Their intermediate-term outcomes are encouraging, with normal graft function. Thus, ABOi-LT may be safe and feasible in HIV-HBV coinfected patients with ESLD. We are cautiously optimistic that ABOi transplantation can be extended to other HIV-positive patients with ESLD.

ARTICLE HIGHLIGHTS

Research background

Human immunodeficiency virus (HIV)-positive patients coinfected with hepatitis B virus (HBV) are eligible for liver transplantation (LT) in Africa and Southeast Asia, particularly China. However, the outcome of HIV-HBV coinfected patients referred for ABO-incompatible LT (ABOi-LT) is unknown.

Research motivation

There have been no reports about the intermediate-term outcome of ABOi-LT in HIV-HBV coinfected recipients.

Research objectives

We sought to clarify the outcome of ABOi-LT for HIV-HBV coinfected patients with end-stage liver disease (ESLD).

Research methods

We report on two Chinese HIV-HBV coinfected patients with ESLD who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. Data of the pre- and post-transplantation were collected, including HIV viral load, CD4(+) T cell count, induction therapy methods, the immunosuppressive regimen and the clinical materials.

Research results

After follow-up for 36-42 mo, both patients survived with undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. Liver biopsy showed no evidence of acute cellular rejection.

Research conclusions

This is the first study of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, which suggests that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

Research perspectives

Due to the relatively small number of cases in the study, follow-up studies with large samples are still required.

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FOOTNOTES

Author contributions: Zhao D and Tang JX performed the conception and design; Zhao D contributed to the administrative support; Tang JX, Zhang KJ and Fang TS contributed to the provision of study materials or patients; Liang ZM, Yan X, Jin X and Zeng XC performed the collection and assembly of data; Weng RH and Xie LJ contributed to the data analysis and interpretation; All authors participated in the writing and final approval of the manuscript.

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Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrollment.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

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CASE REPORT

Eosinophilic enteritis requiring differentiation from chronic enteropathy associated with *SLCO2A1* gene: A case report

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Abstract

BACKGROUND

Eosinophilic gastrointestinal disease (EGID) is a disorder characterized by infiltration of eosinophils causing mucosal damage and dysfunction of the gastrointestinal tract. The endoscopic findings of eosinophilic enteritis (EoN), an EGID variant, are nonspecific and occasionally difficult to diagnose. In contrast, chronic enteropathy associated with *SLCO2A1* (CEAS) is a chronic persistent small intestinal disorder characterized by endoscopic findings such as multiple oblique and circular ulcers.

CASE SUMMARY

We report the case of a 10-year-old boy who had suffered abdominal pain and fatigue for the preceding 6 mo. He was referred to our institute for investigation of suspected gastrointestinal bleeding because of severe anemia with hypoproteinemia and positive fecal human hemoglobin. The upper and lower gastrointestinal endoscopic findings were normal; however, double-balloon small bowel endoscopy showed multiple oblique and circular ulcers with discrete margins and mild constriction of the intestinal lumen in the ileum. The findings were highly consistent with CEAS, but urine prostaglandin metabolites were within normal limits, and no previously reported mutations in the *SLCO2A1* gene were identified. Histological evaluation demonstrated moderate to severe eosinophilic infiltration localized to the small intestine suggesting a diagnosis of EoN. Clinical remission was maintained with montelukast and a partial elemental diet, but emergent surgery for bowel obstruction due to small intestinal stenosis was performed two years after the initial treatment.

CONCLUSION

EoN should be considered in the differential diagnosis of CEAS-like small intestinal ulcerative lesions and normal urinary prostaglandin metabolite levels.

Key Words: Anemia; Chronic enteropathy associated with *SLCO2A1*; Double-balloon endoscopy; Eosinophilic gastrointestinal disease; Hypoproteinemia; Case report

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Core Tip: Eosinophilic enteritis (EoN), a form of eosinophilic gastrointestinal disease localized to the small intestine, is extremely rare in children. The present pediatric case of EoN displayed multiple ulcerative lesions mimicking chronic enteropathy associated with *SLCO2A1* and bowel obstruction due to small intestinal stenosis. The diagnosis was confirmed by small intestinal biopsy using double-balloon enteroscopy and analysis of urine prostaglandin metabolites.

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INTRODUCTION

In eosinophilic gastrointestinal disease (EGID), tissue and functional disorders of the gastrointestinal tract are caused by inflammation due to abnormal infiltration of eosinophils within the gastrointestinal wall[1]. EGID can occur in any location between the esophagus and the colon, but localization to the small intestine is extremely rare[2]. The disease was re-classified from eosinophilic gastroenteritis (EGE) to eosinophilic enteritis (EoN) in 2022[3]. In addition, the nonspecific gastrointestinal endoscopic findings of EGID (edema, erythema, erosions, and ulcers) lead to difficulty in differentiating EGID from other digestive disorders[4].

In contrast, chronic enteropathy associated with *SLCO2A1* (CEAS) is a chronic persistent small bowel disease characterized by multiple oblique and circular ulcers with discrete margins in the ileum endoscopically. It is complicated by small intestinal obstruction due to ulcerative scarring and stenosis in the natural course[5].

Herein, we report a pediatric case of EoN involving multiple ulcerative lesions mimicking CEAS with diagnostic and therapeutic difficulties.

CASE PRESENTATION

Chief complaints

A 10-year-old Japanese boy presented to his family pediatrician with the complaints of easy fatigue and abdominal pain for 6 mo.

History of present illness

The patient presented to the family pediatrician with facial pallor and severe anemia (Hb: 2.9 g/dL) and was referred to his previous physician for admission. He then received red blood cell transfusion and iron supplementation. Further analysis also showed positive fecal human hemoglobin, indicating anemia due to gastrointestinal bleeding, and the patient was transferred to our institution for further evaluation.

History of past illness

The patient had no previous medical history.

Personal and family history

Prior to the patient's birth, the father had been treated with antibiotics for iron deficiency anemia caused by *Helicobacter pylori* infection.

Table 1 Laboratory findings on admission

	Laboratory data	Reference range
White blood cell count (/μL)	5300	4000-8000
Differential (percent)		
Neutrophils	2597 (49.0)	1800-4800
Lymphocytes	2067 (39.0)	1000-3600
Eosinophils	53 (1.0)	40-400
Hemoglobin (g/dL)	9.7	14-18
Hematocrit (%)	35.0	40-48
MCV (fL)	74.9	84-99
MCHC (g/dL)	27.7	32-36
Ferrum (μg/dL)	39	50-190
Ferritin (ng/mL)	23	30-400
Total protein (g/dL)	6.1	6.7-8.3
Albumin (g/dL)	2.9	3.9-4.9
Creatinine (mg/dL)	0.34	0.61-1.04
C-reactive protein (mg/dL)	0.1	< 0.3
Erythrocyte sedimentation rate (mm/h)	4	0-15
IgG (mg/dL)	665	870-1700
IgE (IU/mL)	515	0-173
Fecal human hemoglobin (ng/mL)	2018	< 50
Fecal calprotectin (μg/g)	510	< 50

MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; IgM: Immunoglobulin M; IgE: Immunoglobulin E.

Physical examination

On physical examination, vital signs were as follows: Temperature, 36.8 °C; blood pressure, 99/60 mmHg; heart rate, 80 beats per min; respiratory rate, 18 breaths per min. His height was 122.7 cm (-1.63 standard deviation), and his weight was 24.1 kg (-0.92 standard deviation), with no significant growth disturbance on the growth curve and no other abnormal physical findings other than pale eyelid conjunctiva.

Laboratory examinations

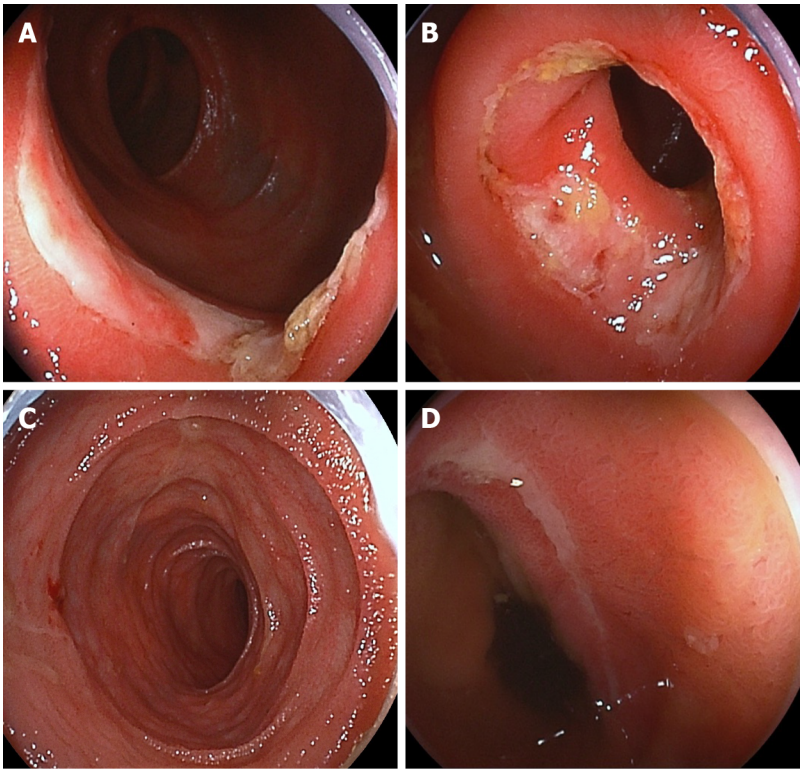
Blood analysis demonstrated low levels of hemoglobin (9.7 g/dL) and albumin (2.9 g/dL), and fecal analysis showed elevated levels of human hemoglobin (2018 ng/mL) and calprotectin (510 μg/g). No elevation of inflammatory markers and no eosinophilia were observed (Table 1).

Imaging examinations

Upper and lower gastrointestinal endoscopy showed normal mucosal findings. Small intestinal capsule endoscopy was not performed because of the patency capsule retention in the stomach, and transanal double-balloon enteroscopy (DBE) was performed. DBE showed multiple oblique and circular ulcers with discrete margins at 70-100 cm proximal from the ileocecal valve with slight constriction of the small intestinal lumen (Figure 1).

Further diagnostic work-up

No histological abnormalities were identified on biopsies conducted by upper and lower gastrointestinal endoscopy. The ileal biopsy with DBE showed moderate to severe histological eosinophilic infiltration [maximum 80 eosinophils/high-power field (HPF)] and cryptitis within the mucosa (Figure 2). Of the urine prostaglandin metabolites that are elevated in CEAS, the levels in the present patient were as follows: Prostaglandin F2α metabolite, 3.2 (normal range: 3.0-4.0) ng/mg Cre; prostaglandin E2 metabolite, 2.09 (normal range: 2.0-3.0) ng/mg Cre; and prostaglandin D2 metabolite, 8.5 (normal range: 9.0-10) ng/mg Cre, all of which were within the normal ranges[6,7]. No previously



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Figure 1 Findings of initial double-balloon small intestinal endoscopy. A: Initial double-balloon enteroscopy (DBE) shows multiple oblique ulcers with discrete margins, 70-100 cm proximal to the ileal valve; B: The circular ulcers with slight constriction of the small intestinal lumen at initial DBE; C: Follow-up DBE performed 1 year later shows mucosal healing; D: The oblique ulcer and scars at 70 cm proximal to the ileal valve at follow-up DBE.

reported mutations in the *SLCO2A1* gene or in the targeted gene panels for very early-onset inflammatory bowel disease were identified[8].

FINAL DIAGNOSIS

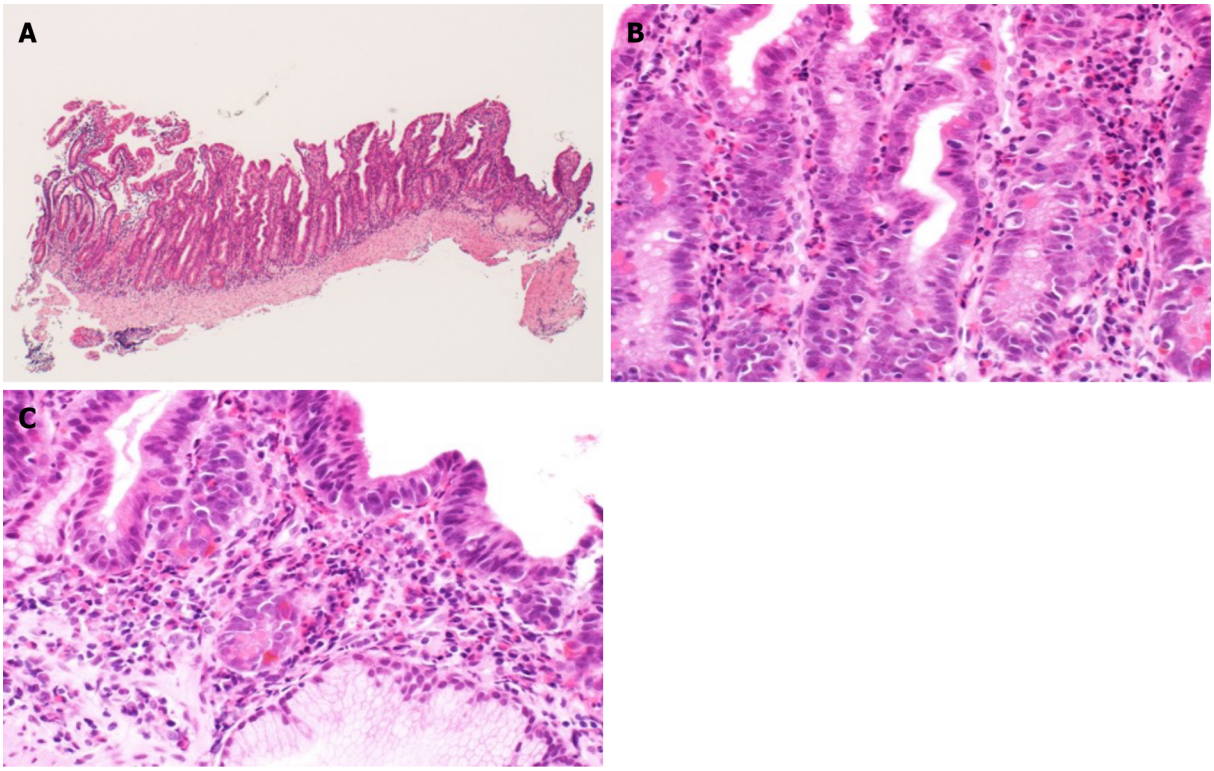
The diagnostic findings and medical history indicated a final diagnosis of EoN.

TREATMENT

The patient was treated with montelukast (10 mg/d for a total of 26 mo), which reduced the frequency of abdominal pain. Partial elemental diet therapy (600 kcal/day for a total of 24 mo) was also implemented due to insufficient response of hypoalbuminemia and anemia[9]. Corticosteroids were not administered because the patient's family preferred that steroids be avoided.

OUTCOME AND FOLLOW-UP

The abdominal pain resolved completely 2 mo after the administration of montelukast and the partial elemental diet, and improvement of hemoglobin (11.2 g/dL) and hypoalbuminemia (3.5 g/dL) and normalization of fecal human hemoglobin (56 ng/mL) were observed after 4 mo. At follow-up of the small intestine by DBE performed 1 year later, mucosal healing was achieved, except for the oblique ulcer and scars at 70 cm proximal to the ileal valve, and no intestinal stenosis caused by the healing ulcer was observed (Figure 1). Eosinophilic infiltration had also disappeared on biopsy, suggesting histological remission. The patient was in clinical remission thereafter, but 2 years and 2 mo after the first visit, sudden bowel obstruction was induced by small intestinal stenosis, and emergent surgery was performed. The ileal macroscopic findings showed strictures at 40 cm and 44 cm proximal to the ileocecal valve, leading to ileal resection of the strictures and ileostomy (Figure 3). The histological findings of the resected specimen were of ulcer formation and peri-ulcer mucosal damage, suggesting intestinal stenosis in the process of ulcerative scarring. No significant granuloma or eosinophilic infilt-



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Figure 2 Histopathological findings of ileal tissue obtained from double-balloon enteroscopy. A: Hematoxylin and eosin staining (× 100) shows mild villous atrophy; B: Hematoxylin and eosin staining (× 400) shows moderate to severe histological eosinophilic infiltration (maximum 80 eosinophils/high-power field); C: Hematoxylin and eosin staining (× 400) shows crypt destruction (cryptitis).

ration was observed (Figure 3). The patient's postoperative course was uneventful. Ileostomy closure was performed 2 mo later, and the patient is currently being followed on an outpatient basis. An ileal resection specimen obtained at ileostomy closure showed marked eosinophilic infiltration (> 50/HPF) in the subserosa (Figure 3).

DISCUSSION

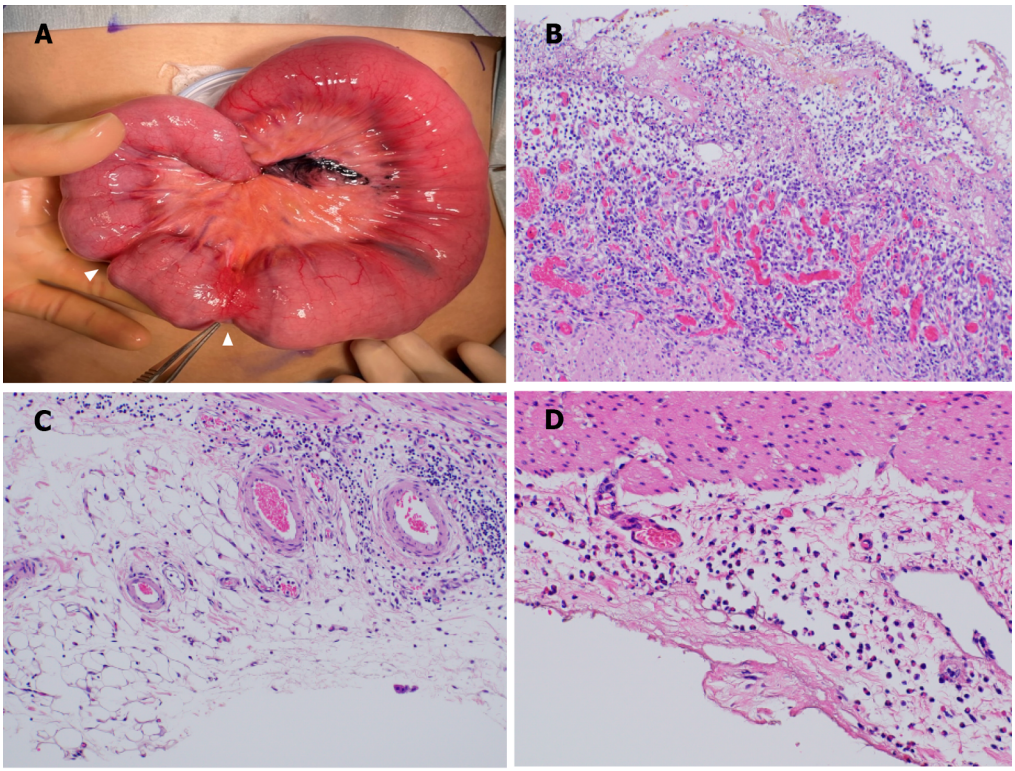
The incidence of EGE in the United States is 2.5-30 cases per 100000 people, whereas the incidence in Japan is estimated to be 5.5 times higher[10]. EGE is usually difficult to diagnose because of the variety of gastrointestinal symptoms, as well as the extremely nonspecific findings of gastrointestinal endoscopy[11]. A case series that reported the small intestinal capsule endoscopic findings in 10 EGE cases found small intestinal lesions such as multiple erythematous lesions in 6 cases, erosions and ulcers in 5 cases, flattened or missing villi in 4 cases, and intestinal stenosis in 7 cases[9]. In that study, EGE was defined as EGID with extensive lesions extending from the stomach to the large intestine; however, only one pediatric and 6 adult cases of EoN localized to the small intestine have been reported (Table 2)[12-17]. These reports described various forms of ulcerative lesions and strictures in the small intestine, but all patients were diagnosed with EGE because of the difficulty of distinguishing EGE from CEAS based on the endoscopic images, as in the present case, and the patients had histologically significant eosinophilic infiltrates[12-17]. In contrast, the present case showed hypoproteinemia and iron deficiency anemia combined with multiple oblique and circular ileal ulcers, consistent with the diagnostic criteria for CEAS[18].

CEAS was first reported in Japan in 1968 as "nonspecific multiple ulcers of the small intestine"[5] and is caused by *SLCO2A1* germline variants encoding a prostaglandin transporter. The identification of hot spots of *SLCO2A1* variants is thus valuable for diagnosis but not currently included in the definitive diagnostic guidelines[5,7,18]. In addition, the clinical manifestations of CEAS are chronic and intractable nonspecific gastrointestinal symptoms comparable to EGID, and no effective treatment for these disorders has been established[18]. Ulcers of CEAS have been described as shallow oblique, circular, or longitudinal with discrete margins in case series of the endoscopic findings of CEAS[13,19]. CEAS-like diseases without *SLCO2A1* mutations have also been reported, including inherited eicosanoid metabolic disorders, inherited human cPLA2α deficiency, and cryptogenic multifocal ulcerous stenosing enteritis; histologically, however, these diseases show nonspecific inflammatory cell infiltration predominantly

Table 2 Summary of four previous cases of eosinophilic enteritis

Case	Age/sex	Location	Endoscopic findings				Laboratory findings	
			Multiple erythema	Erosions	Ulcer	Stricture	Anemia	Hypoproteinemia
1	62/F	Throughout	-	+	+	-	NR	NR
2	66/F	Throughout	-	+	+	-	NR	NR
3	48/M	Upper jejunum/ileum	-	-	+	-	-	-
4	2/M	Jejunum/proximal ileum	+	-	-	-	-	NR
5	70/F	Ileum	-	-	-	-	-	+
6	54/M	Ileum	-	-	-	+	-	-
7	68/M	Distal jejunum/proximal ileum	-	-	-	+	-	-

F: Female; M: Male; NR: No record.



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Figure 3 Macroscopic and microscopic histological findings of the resected ileum. A: Macroscopically, strictures are observed 40 cm and 44 cm proximal to the ileocecal valve (arrowhead); B: Resected ileum shows ulcer formation and peri-ulcer mucosal damage histologically; C: No significant eosinophilic infiltration is observed transmurally; D: Resected ileum at the ileostomy closure shows marked eosinophilic infiltration (> 50/high-power field) in the subserosa.

by neutrophils, rather than eosinophils[20,21]. The present case thus meets the diagnostic criteria for CEAS, but the diagnosis of EoN was reasonable based on the histological findings and the therapeutic course and responsiveness.

The patient developed bowel obstruction induced by small bowel stricture during the clinical course. Most cases of CEAS manifest with small bowel stricture associated with the healing of ulcers and require long-term endoscopic follow-up and treatment, whereas few cases of small bowel stricture have been reported in EGID[16,17,22,23]. In particular, two EoN cases with stricture showed a transmural eosinophilic infiltration at the resected intestinal tract[16,17], suggesting that the eosinophilic infiltration in the muscle layer and serosa is a risk factor for intestinal stricture. However, identification of these by endoscopic mucosal biopsy may be extremely difficult. In the present case, the mucosal eosinophilic infiltration at the time of the small bowel resection was insignificant compared to that at the ileostomy closure, in which insufficient therapeutic agents were regularly administered, suggesting the treatment for EoN was unlikely to be inadequate. This fact indicates that periodic endoscopic follow-up with

consideration of the possibility of small bowel stricture would be required in EoN localized to the small bowel with CEAS-like ulcerative lesions.

CONCLUSION

EoN should be included in the differential diagnosis of patients who exhibit CEAS-like ulcerative lesions localized to the small intestine and have normal urinary prostaglandin metabolites. In addition, EoN with CEAS-like ulcerative lesions may require periodic endoscopic follow-up taking into account the potential complication of small bowel stricture.

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FOOTNOTES

Author contributions: Kimura K, Jimbo K, and Arai N contributed to manuscript writing and editing, and data collection; Jimbo K, Sato M, and Suzuki M contributed to data analysis; Kudo T, Yano T, and Shimizu T contributed to conceptualization and supervision; all authors have read and approved the final manuscript.

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REVIEW

Mitochondrial carnitine palmitoyltransferase-II dysfunction: A possible novel mechanism for nonalcoholic fatty liver disease in hepatocarcinogenesis

Min Yao, Ping Zhou, Yan-Yan Qin, Li Wang, Deng-Fu Yao

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Abstract

Nonalcoholic fatty liver disease (NAFLD) or metabolic-associated fatty liver disease has been characterized by the lipid accumulation with injury of hepatocytes and has become one of the most common chronic liver diseases in the world. The complex mechanisms of NAFLD formation are still under identification. Carnitine palmitoyltransferase-II (CPT-II) on inner mitochondrial membrane (IMM) regulates long chain fatty acid β-oxidation, and its abnormality has had more and more attention paid to it by basic and clinical research in NAFLD. The sequences of its peptide chain and DNA nucleotides have been identified, and the catalytic activity of CPT-II is affected on its gene mutations, deficiency, enzymatic thermal instability, circulating carnitine level and so on. Recently, the CPT-II dysfunction has been discovered in models of liver lipid accumulation. Meanwhile, the malignant transformation of hepatocyte-related $CD44^+$ stem T cell activation, high levels of tumor-related biomarkers (AFP, GPC3) and abnormal activation of Wnt3a expression as a key signal molecule of the Wnt/β-catenin pathway run parallel to the alterations of hepatocyte pathology. This review focuses on some of the progress of CPT-II inactivity on IMM with liver fatty accumulation as a possible novel pathogenesis for NAFLD in hepatocarcinogenesis.

Key Words: Carnitine palmitoyl transferase-II; Nonalcoholic fatty liver disease; Fatty acid β-oxidation; Carnitine; Hepatocyte malignant transformation; Mitochondrial membrane

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Core Tip: The complex mechanisms of nonalcoholic fatty liver disease formation are still under identification. Hepatic carnitine palmitoyl transferase-II (CPT-II) on inner mitochondrial membrane regulates long chain fatty acid β-oxidation and this abnormality has had more attention paid to it by basic and clinical research. The sequences of its peptide chain and DNA nucleotides have been identified and the catalytic activity of CPT-II is affected on its gene mutations, deficiency, enzymatic thermal instability, circulating carnitine level and so on. CPT-II dysfunction has been discovered in models of lipid accumulation. Meanwhile, the malignant transformation of hepatocyte-related $CD44^+$ stem T cell activation, high levels of tumor-related biomarkers and abnormal Wnt3a expression as a key signal molecule of the Wnt/β-catenin pathway run parallel to the alterations of hepatocyte pathology.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) or metabolic-associated fatty liver disease (MAFLD) is a general term of liver diseases characterized by inflammation, fatty accumulation and hepatocyte dysfunction, except of alcohol or other clear liver injury factors[1-3]. Up until now, NAFLD has become a potentially serious liver disease that affects approximately 25% of the adult population in the world [4], and is divided into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) with or without liver fibrosis[4,5]. It has been shown that balloon like hepatocyte injury is based on NAFL. Most patients have no obvious symptoms and may not be diagnosed until they develop into liver cirrhosis or progress to hepatocellular carcinoma (HCC), and the effect of early clinical screening is poor [6]. Once NAFLD progresses to liver cirrhosis, it is difficult to reverse and there is a risk that HCC can't be ignored. Not only that, it also involves the occurrence of multiple systemic diseases in the body which are closely related to cardiovascular disease, chronic kidney disease and colorectal tumor which all threaten human health[7,8]. Therefore, finding the monitoring target in the malignant transformation of NAFLD has practical clinical significance for the prevention of NAFLD-related liver malignant diseases[9].

Lipid metabolism rearrangements in NAFLD contribute to disease progress that has emerged as one of the most risks for HCC, where metabolic reprogramming is a hallmark[10]. Hepatic carnitine palmitoyl transferases (CPTs) are critical for long-chain fatty acids (LCFAs) -oxidation, as they are capable of transport through the mitochondrial membrane[11]. CPT is made up of two separate proteins (CPT-I and CPT-II). CPT-I is located in the outer mitochondrial membrane (OMM) with three isoforms (liver CPT1a, muscle CPT1b and brain CPT1c) and CPT-II is in the inner mitochondrial membrane (IMM)[12,13]. The amino acid and cDNA nucleotide sequences of the ubiquitous CPT-II have been elucidated. The mutations or dysregulation of the CPTs, which are associated with many serious and even fatal diseases, are promising targets for developing drugs to treat type 2 diabetes (T2D) and obesity[14,15]. Dysregulated lipid metabolism is involved in human diseases, including chronic inflammatory diseases and inflammatory-related tumors[16]. CPTs play an important role in lipid metabolism and fatty acid oxidation (FAO) in mitochondria. CPT-II has been confirmed as a rate-limiting enzyme and in regulation of host immune responses[17,18]. However, the pathological role of CPT-II alteration with NAFLD remains to be identified[19]. This review summarizes the latest research findings of CPT-II, which are important for accurate or early monitoring of NAFLD malignant transformation.

CPT2 STRUCTURE

The most important function of the *CPT* family is to ensure that fatty acids enter the mitochondria for -oxidation. Transmembrane protein CPT-I is located in OMM and CPT-II is in IMM. Human CPT-II gene (*CPT2)* as an autosomal recessive trait encoded gene localizes on chromosomes 1 (1p32) and the gene

full length contains 3090 nucleotides and 5 exons, which can encode the enzyme protein peptide chain composed of 658 amino acids[20]. Summaries of *CPT2*, CPT-II and total numbers of its reported mutated sites are shown in Table 1. Human CPT-II (NM_000098) is a mitochondrial protein in IMM. CPT-II together with CPT-I oxidize LCFA in the mitochondria and play pivotal roles in the LCFA transport across the mitochondrial membrane for β-oxidation[21]. In molecular genetic aspects, *CPT2* is identified in about 70% of mutant alleles. There are variations in the *CPT2* genome, most of which are single-base substitutions, small insertions or discrete deletions[22,23]. Among the enzymatic system, CPT-II plays a rate-limiting role in the entry of fatty acids into mitochondrial FAO and is considered to be a key component of cellular metabolic homeostasis[24]. Anti-cancer drug oxaliplatin can activate CPT-II in gastrointestinal cancer cells and promote the catabolism of fatty acids[18]. Knocking down of *CPT2* by patient-derived xenograft models confirmed the regulating role of mitochondrial FAO in Src activation and metastasis of breast cancer[25]. However, a subset of substitutions, insertion or deletion, tend to cluster in all exons, especially in exon 4 and exon 5, suggesting that CPT2 clustering is due to a combination of factors such as the rate of heterogeneous mutations in the genome, biophysical characteristics of exogenous carcinogens, endogenous dysregulation and large mutation events related to genome instability[26].

The enzymatic system that facilitates the transfer is known as CPT mainly in OMM or IMM and plays an important role in maintaining its structural and functional integrity. Liver cells must keep related metabolic homeostasis in a wide range of conditions and meet their ATP needs depending on FAO[27,28]. CPT-II catalyzes transesterified acylcarnitine's transferred from cytosol into the intermembrane space (IMS) and the remaining acyl of acylcarnitine is changed back to CoA on IMM, which is next available for FAO. Meanwhile, the released carnitine is returned to the IMS of the mitochondrion *via* CACT and is available for fatty acid re-transport[29]. However, the deficiency or gene mutation of CPT-II can significantly affect mitochondrial FAO. Bezafibrate, as a well-known hypolipidemic drug, was tested to stimulate CPT2 mutation, but it should be a challenge to restore normal LCFA oxidation from a series of other fatty acid mitochondrial diseases[30,31], indicated that CPT-II not only provides ATP for liver cells *via* FAO, but also its down-regulated expression affects the growth and malignant transformation of hepatocytes *via* cell damage, related signal molecules, stem cells, immunology and so on[32]. Therefore, the study of CPT-II will help to understand the pathogenesis and to develop a promising treatment of NAFLD.

Previous studies of *CPT2* mutations have identified the presence of single-base substitutions, and many other events such as double-base and multiple-base substitutions, insertions or deletions. The reported mutations among all five exons of *CPT2* and 89 mutated sites are shown in Table 2[33,34]. Most of *CPT2* or CPT-II mutations are located in exon 4 or exon 5. Biochemical consequences of these mutations are still controversial. The c.338 C>T (P.S113L) variant can be detected in most cases of Caucasians; in Japanese, c.1148 T>A (P.F383Y) is the most frequent variant allele and can obviously cause severe infant forms of symptoms. Among them, it may include deficiency of enzyme protein, enzyme inactivity or abnormality of enzymatic regulation. The protein encoded by this gene is a nuclear protein which is transported to the IMM. Due to the low activity, thermal instability, and short half-life of CPT-II, the CPT II variant exerts a dominant negative effect on homologous tetrameric proteins associated with mitochondrial LCFA oxidation impairment[35]. Recently, based on animal models or clinical studies, the crystal structures of CPT-II were determined in uninhibited forms and in complexes with inhibiting substrate analogs with anti-diabetic features. The crystal structures have a deep understanding of the enzymatic structure-function relationship which is conducive to the discovery of new inhibitors through structure-based drug design[36].

MITOCHONDRIAL CARNITINE SHUTTLE SYSTEM

Using fatty acids as ATP requires more than 20 enzymes and transporters, which are involved in the activation and transport of fatty acids into the mitochondria. The membrane transport system of fatty acid β-oxidation in mitochondria is shown in Figure 1. Mitochondrial FAO is one of the major pathways for fatty acid degradation and is critical for maintaining ATP balance in the human body[37,38]. When the glucose supply is limited, fatty acids are an important source of energy after absorption and during fasting. But even when glucose is sufficient, FAO still is the main source of energy for human tissues. A series of enzymes, transporters and other facilitating proteins with biochemistry and physiological functions are involved in FAO (Figure 1). The role of CPT in the LCFA oxidation, and this system includes CPT-I, carnitine-acylcarnitine translocase (CACT) and CPT-II. The acyl-CoA synthetase located in OMM catalyzes fatty acids to form acyl-CoA with ATP and CoA participation, and then, transports long-chain acyl-CoA by the delivery system into mitochondria, that is a carnitine shuttle system to enter the process for β-oxidation. Most genes encoding CPT-II are known to be recessive genetic defects and the clinical manifestations of the related diseases may include hypoglycemia, cardiomyopathy, arrhythmias and rhabdomyolysis; It also illustrates the importance of FAO during fasting and in liver and (heart) muscle function[39,40].

Table 1 Summary of *CPT2* gene and reported mutation sites

Exon	Size in bp	Nucleotides	Amino acids	Mut. no.	Mutation sites	
					Amino acids	Others
1	668	1-668	1-51	5	2	3
2	81	669-749	52-78	4	2	2
3	107	750-856	79-113	5	3	2
4	1305	857-2161	114-548	59	49	10
5	929	2162-3090	549-658	16	12	4

Mut no.: Total numbers of nucleotide mutation including insertion, delete or peptide variation from codon substitution; Others: Mutations from double or more nucleotide insertion, deletes and so on.

Table 2 List of known human carnitine palmitoyltransferase-II mutated sites[33,34]

Exon	Amino acid substitution	Others
1	Pro41Leu; Pro50His	36-38 insGC; 6_43dupGGGCCC; 113_114dupGC
2	Pro55Arg; Ala67Gly	182_203del 22; 153-1G>A (Intron 2)[1]
3	Cys84Arg; Ala101Val; Ser113Leu	256_257delAG; 232+1G>A (Intron 3)[1]
4	Tyr120Cys; Leu121Gln; Arg124Gln; Arg124Ter; Asn146Thr; Arg151Gln; Arg151Trp; Arg161Trp; Lys164Ter; Arg167Gln; Pro173Ser; Glu174Lys; Tyr210Asp; Asp213Gly; Met214Thr; Gln216Arg; Pro227Leu; Arg231Trp; Arg247Trp; Lys274Met; Arg296Gln; Arg296Leu; Arg296Ter; Gly310Gly; Cys326Tyr; Asp328Gly; Met342Thr; Phe352Cys; Val368Ile; His369Gln; Arg382Lys; Phe383Tyr; Gln413Gln; Phe448Leu; Arg450Ter; Gly451Glu; Glu454Ter; Lys457Ter; Tyr479Phe; Tyr479Cys; Gly480Arg; Glu487Lys; Gly497Ser; Ile502Thr; Arg503Cys; Pro504Leu; Phe516Ser; Glu545Ala	1569_1570delCA; 1444_1447delACAG; 1634_1636delAAG; 1646_49del; 1273_1274delAC; 1238_1239delAG; 1543_1546delGCCT; 907_918ins11; 533insT; 534-558del25; 1645+5G>A (Intron 5)[1]
5	Arg560Gln; Leu575Pro; Asp576Gly; Ser588Cys; Ser590Asn; Gly600Arg; Pro604Ser; Val605Leu; Asp608His; Tyr628Ser; Arg631Cys; Leu644Ser	1816_1817delGT; 1923_1935del; 340+1G>A (Intron 4)[1]; 340+5G>A (Intron 4)[1]

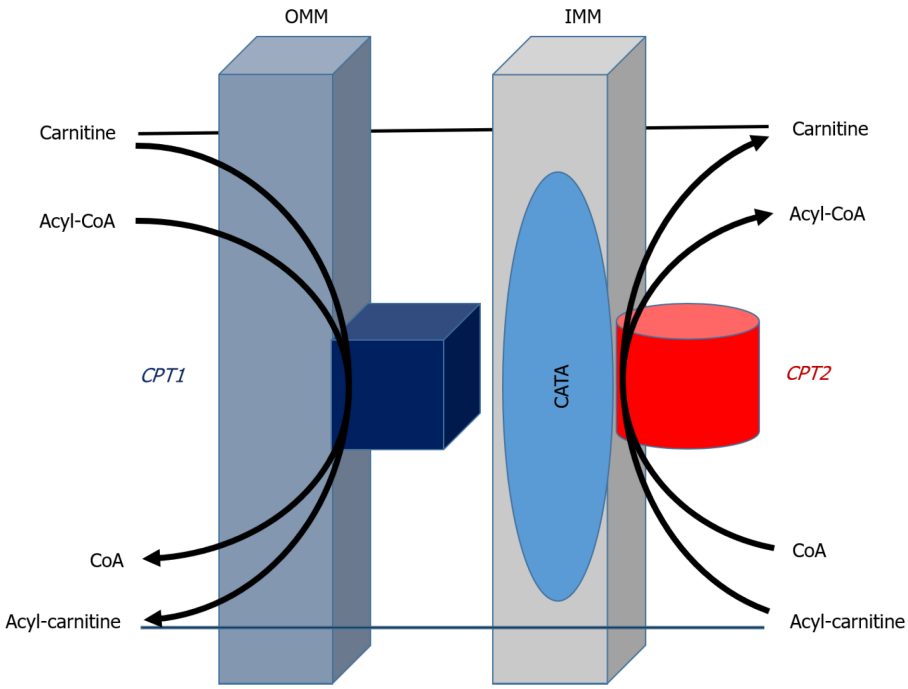
[1]Intron.

Mutations are listed on Human Gene Mutation Database from Stenson *et al*[33] and Yao *et al*[30].

The carnitine shuttle system controls fatty acid translocation across the mitochondrial membrane. Key enzymes determine the competition of glycolysis *vs* mitochondrial FAO defined by the Randle cycle. This transport system with CACT is an important part of fatty acid esterification through OMM and IMM of mitochondria (Figure 1). First, CPT-I at OMM catalyzes long-chain acyl-CoA along with carnitine conversion to long-chain acylcarnitine and CoA is transported to the mitochondrial interior with help of translocase on the mitochondrial intima[41,42]. After that, the transesterified acylcarnitine's are transported from the cytosol into the IMM space as CACT acylcarnitine releases carnitine by CPT-II catalysis, and converts it to an acyl group from carnitine to acyl-CoA, available for β-oxidation. Released carnitine returns to the IMM space of the mitochondria for fatty acid re-transport[43]. CPT-II plays an important regulatory role in FAO and its function affects fatty acid metabolism. More importantly, CPTs in the carnitine shuttle system can be used as a drug target to reduce gluconeogenesis or restore liposome balance. Therefore, it has the potential value of gene therapy or immunotherapy, and the further study of the mechanism of CPTs could provide useful ideas for clinical treatment of related diseases[44,45].

EFFECT FACTORS OF CPT-II ACTIVITY

Lipid metabolism involves a variety of biological processes, including the most important lipid metabolic pathway (FAO with carnitine shuttle system). The mutations or dysregulation of hepatic CPT-II have been linked to many serious or even fatal human diseases, and it should be a promising target for developing drugs to treat T2D or obesity[46]. However, the deficiency, over-expression or inactivation of liver CPT-II might ultimately lead to disruption of immune homeostasis, thereby increasing the risk of various inflammatory diseases and even tumors. There is some evidence that CPT-II or the associated mitochondrial LCFA are involved in the development and progression of these related diseases. Thus, the agonists or inhibitors targeting the CPTs or carnitine shuttle system have



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Figure 1 Transport system of fatty acid oxidation in outer mitochondrial membrane and inner mitochondrial membrane[11,65]. Fatty acid β-oxidation is catalyzed by enzymes located in outer and inner mitochondrial membrane to form acyl-coenzyme A (CoA) in the participation with ATP and CoA. Carnitine palmitoyltransferase (CPT)-I promotes the conversion of acyl-CoA to acyl-carnitine that is transported to mitochondrial interior with the help of translocase on the intima of mitochondria. Under the catalysis of CPT-II, acylcarnitine releases carnitine, and then converted to acyl-CoA to enter β-oxidation. The CPT system with carnitine acyl-carnitine translocase play a vital part in the transport system for esterification of fatty acids through the mitochondrial membrane and CPT-II as a key rate-limiting enzyme for fatty acid β-oxidation. OMM: Outer mitochondrial membrane; IMM: Inner mitochondrial membrane; CACT: Carnitine acyl-carnitine translocase; CPT: Carnitine palmitoyltransferase; CoA: Coenzyme A.

emerged as novel therapies for these diseases[47]. Normal function of FAO in IMM is closely dependent on the catalytic activity of CPT-II that could be affected by *CPT2* variation, the amino acid substitution of enzyme, inhibition of enzyme activity, circulating carnitine level and so on.

CPT-II deficiency

Hepatic CPT-II deficiency is one of the most common forms of mitochondrial FAO disorders (FAODs) and have several clinical presentations that have been known for a long time. However, its phenotypic variability remains fascinating[48]. The clinical phenotypes of CPT-II deficiency are classified into muscular, severe infantile and fatal neonatal types. In addition, neonatal-onset CPT-II deficiency is often accompanied by brain and kidney organ dysfunction features, such as in the 1st mo of life, and is almost always fatal. Three different phenotypes (neonatal, infant and adult onset) have been identified, all with autosomal recessive inheritance patterns[49]. The clinical phenotype of adult CPT-II deficiency is mostly benign, and only with additional external stimuli, such as high-intensity exercise, can lead to major myopathy symptoms. However, the perinatal and infantile CPT-II deficiency usually involves multiple organ systems, especially when occurring in the perinatal period as it is the most serious form and is often fatal[50]. The application of mass spectrometry technology to analyze acylcarnitine profiles in blood has revolutionized the FAOD diagnosis, including CPT-II deficiency. In most cases, the number of *CPT2* mutations is increasing and there is a clear genotype-phenotype correlation. However, the clinical variants in some patients might contain other genetic or environmental factors[51].

In the clinical setting, the manifestations of patients with CPT-II deficiency include severe infant liver disorders, myocardial infarction, fatality in neonates and myopathy (usually mild, from infancy to adulthood). Some patients have serious multi-system disease that includes liver function failure, cardiomyopathy, epilepsy, hypoglycemia and premature death, while others are characterized by muscle pain and weakness which is sometimes accompanied by myoglobinuria[52]. The proband was diagnosed for CPT-II deficiency by finding a decrease in muscle CPT activity or by identifying a biallelic variant of *CPT2* in a molecular genetics test. A total of six mutations have been identified, including four new ones. Among those mutations, the S113 L mutation is common in about 50% of the mutant alleles. Three of the six mutations (3/6) have been found in a few unrelated patients, while others have been found in only one family with genetic heterogeneity. To date, about 100 *CPT2* mutations have been discovered. Prenatal diagnosis is provided when the risk of infant/severe CPT-II deficiency is 1/4. Infantile CPT-II presents as a severe hypoglycemic episode of ketoacidosis, occasionally associated with

heart damage and usually resulting in sudden death before age 1[50]. Treatment for *CPT2* deficiency includes a low-fat diet in rich triglyceride or carnitine and avoiding fasting or hyperkinesis[53].

Thermal instability of CPT-II

CPT-II activation is associated with disorders of mitochondrial β-oxidation of LCFA in IMM. Based on the crystal structure of mouse CAT, the active site of CPT-II is located at the interface between two domains, extending in tunnels through the enzyme protein centers, alone or in complex with its substrate carnitine or CoA[54]. In this tunnel, carnitine combines with CoA and its opposite is catalytic His^{343} residue. The information of CPT-II structure provides a molecular basis to understand the catalytic activity of CAT or to design their inhibitors. In addition, the carnitine might contribute to the catalytic stabilization of oxygen ions in the reaction intermediates. Hepatic CPT-II is sensitive to inhibition by metabolites of fatty acids, Triton X-100, or malonyl-CoA[55].

Artificially recombinant His6-N-hCPT2 and His6-N-hCPT2/S113L showed the same enzymatic activity for wild-type or S113L variants of CPT-II[56]. However, the mutant CPT-II exhibited abnormal destabilization at 40 °C or 45 °C and was more sensitive to be inhibited by malonyl-CoA. The thermal solubility of mutant CPT-II, which may explain the symptoms of CPT-II deficiency may mainly occur during prolonged exercise, infection, and exposure to cold. In addition, CPT II abnormalities are likely to be largely suppressed when fatty acid metabolism is stressed[54]. The unstable CPT II variants with enzymatic inactivity might lower mitochondrial fuel utilization under the phenotypic threshold during patients with hyperthermia, thus suggesting that hepatic CPT-II should play a pathological role in NAFLD progression.

High-risk patients have thermolabile genetic backgrounds of CPT-II in LCFA metabolism. However, until now, no related mutation of CPT-II was reported in NAFLD patients[57,58]. Almost fatal or handicapped virus-associated encephalopathy cases exhibited transiently higher serum LCAC levels during fever more than 40 °C. The specific activity of patients' CPT-II (0.4 ± 0.06 nMol/min/mg) was 36% of normal control (1.1 ± 0.3 nMol/min/mg protein) at 37 °C. The CPT-II specific activity in the patient group was down to 50% for 2 h at 41 °C, and CPT-II in the normal control group still was 91.4%, and the sequencing analysis of patients' *CPT2* gene revealed compound (1055T>G/F352C) + (1102G>A/V368I) heterozygous variations[46,59]. F352C substitution was only reported in the Japanese study, and V368I polymorphic variation has relatively mild effects related to CPT-II deficiency[47,60]. The CPT-II mutation or dysregulation has been linked to more serious, even fatal diseases, and these data should be promising molecule targets to develop therapeutic agents for NAFLD in future.

Carnitine level

Carnitine as a substance has a wide range of biological functions, including transport of LCAD from the cytoplasm to the mitochondrial matrix, regulation of acetyl-CoA/CoA, control of acyl transport between organelles and prevention of oxidative stress[58]. Maintaining normal fat metabolism depends on carnitine concentration that is synthesized in most eucaryotic organisms[61]. The methylation of lysine initiates the biosynthesis of carnitine. The formed trimethyllysine is then converted to butylbetaine in all tissues and finally hydroxylated to carnitine in the liver and released from the tissues, which are then actively absorbed by all other tissues[62,63]. This transfer requires the enzyme and transporter that accumulates carnitine within the cell (OCTN2 carnitine transporter), which is conjugated to LCFA (CPT-I), to transfer acylcarnitine's through IMM (CACT), and to transfer carnitines through the IMM (CACT), fatty acids were conjugated back to CoA for subsequent -oxidation (CPT-II). The regulation of carnitine synthesis is still incompletely understood because the turnover of carnitine in the human body is slow[64].

Carnitine is essential for proper fat metabolism, producing ATP, and the transport of LFAC or medium fatty acid chains (MFAC). It attracts LFAC and MFAC, after it breaks them down, and then takes them to the cell's mitochondria for FAO. And as it turns out, the body burns more fat, providing the body with more natural energy in the process[65,66]. According to the previous study, using a carnitine antagonist 3-(2,2,2-trimethylpropionate hydrazine dihydrate, THP) resulted in lipid accumulation with increased liver weight in wild-type mice. The competition between THP and carnitine inhibited CPT-II activity, resulting in carnitine deficiency, acyl CoA and fat accumulation[67]. Clinical data showed that the blood carnitine concentration in NAFLD patients was lower than those in healthy people, and the level in NAFLD cases with liver cirrhosis accounted for only 22% of normal people[68,69]. The concentration of carnitine in patients with liver disease is low, the fat accumulation in rat liver tissue, the content of total fatty acids, free fatty acids, short chain fatty acids (SCFC) and LCFA in liver, and the content of chain, long chain, short chain and total fatty acids in circulating blood also change. During patients with hepatitis B or hepatitis C virus infection, or with mitochondrial FAODs present with NAFLD or severe liver diseases, enough carnitine should play an important role in the mitochondrial carnitine shuttle system, suggesting that circulating carnitine level affects FAO, ameliorates mitochondrial dysfunction, reduces insulin resistance and improves NAFLD progression [70,71].

CPT-II INACTIVITY IN NAFLD

NAFLD pathogenesis is much more complicated with multi-factorial events. Recently, the low activity of CPT-II on IMM during NAFLD progression has attracted much attention both in basic and clinical aspects[72,73]. Although many theories of NAFLD with abnormal lipid metabolism[8,74,75] such as insulin resistance (IR), lipid peroxidation, cytokine expression, iron overload, genetics, environment, immunity, drugs, living habits and so on. However, there are still many problems in the study of NAFLD pathogenesis. According to these theories, the IR stimulates liver fat accumulation and triglycerides, resulting in the first strike to NAFLD formation; then oxidative stress and lipid peroxidation aggravate hepatocyte injury to develop into the second strike that starts with asymptomatic steatosis, and continues to cell inflammation, steatohepatitis, fibrosis or hepatocyte malignant transformation[76,77]; hence a "multiple hit" hypothesis seems a more accurate proposal[78,79]. Up to now, the new discovery of loss of CPT-II activity has been confirmed in lipid accumulating models that should be one of the NAFLD mechanisms.

Ideal NAFLD models should correctly reflect both histopathology and pathophysiology, and imitate certain aspects of NAFLD which are divided into genetic, dietary and combination models referring to advantages and disadvantages[80,81]. Also, the models based on biological knowledge are reliable and reproducible, having low mortality, and being compatible with simple and feasible methods, not only in elucidating pathogenesis for understanding NAFLD but also in examining therapeutic effects of various agents to develop tools and giving crucial information. Inhibiting CPT-II activity is related to a disorder of lipid metabolism, which may be related to NAFLD pathogenesis and down-regulating CPT-II in liver tissues. Gene defects are associated with mitochondrial LCFA oxidation disorders[82,83].

In order to determine the independent and interdependent roles of triglyceride (TG) hydrolysis and FAO, liver-specific defects in mice were generated in TG hydrolysis (*AtglL*$^{-/-}$) , FAO (*CPT2L*$^{-/-}$) , or both (double knockout)[73,84]. Loss of a single component of FAO [*CPT2*, adipose TG lipase (*Atgl*), and peroxisome proliferators-activated receptor-α (PPAR-α)] resulting in a major independent effect on the morphology of liver cells, gene expression, and intermediate metabolism in response to fasting[84]. However, the mice in the high-fat diet (HFD) model revealed an interdependent role for *Atgl* and *CPT2*, as deletion of only one gene lead to NAFLD; But loss of both components leads to significant hepatocyte inflammation and liver fibrosis[85].

CPT-II IN HEPATOCARCINOGENESIS

During NAFLD progression, the transcription factors E2f1 and E2f2 contributed to NAFLD-associated mice HCC and their involvement in metabolic recombination[72,85]. The expressions of E2f1 and E2f2 were significantly increased in the NAFLD-associated HCC in mice induced with HFD plus diethylnitrosamine (Den). However, the *E2f1*$^{-/-}$ and *E2f2*$^{-/-}$ mice were resistant to DEN-HFD-induced hepatocarcinogenesis and were associated with lipid accumulation. The administration of DEN-HFD in the *E2f1*$^{-/-}$ and *E2f2*$^{-/-}$ mice enhanced FAO and increased expression of *CPT2* because of CPT2 as an essential enzyme for FAO, whose down-regulation was linked to the NAFLD-related hepatocarcinogenesis[86]. The mouse models of obesity-driven and NASH-driven HCC typically exhibit robust steatosis in HCC cells, as seem to be seen in the human NASH-HCC. The livers and HCC tissues from diethylnitrosamine-injected mice fed either control or HFD were subjected to comprehensive metabolome analysis[80,87]. Extensive acylcarnitine's accumulation was seen in liver cancer tissue and in the sera of HFD-fed mice. A similar increasing level was seen in sera from patients with NASH-associated liver cancer. The increase of acylcarnitine might be related to the CPT-II down-regulation, suggesting that acylcarnitine is a surrogate marker of the down-regulation of CPT-II and directly participates in the development of hepatocarcinogenesis.

The down-regulation of CPT-II caused FAO inhibition which might be the cause of steatosis in HCC. The knockdown of the *CPT2* gene in HCC cells could inhibit the Src-mediated activation of JNK and produce anti-lipotoxicity. Furthermore, oleylcarnitine promotes spheroid formation in HCC cells through STAT3 activation[88,89]. HFD feeding and carnitine supplementation synergistically enhance hepatocarcinogenesis with acylcarnitine accumulation *in vivo*. The CPT-II level in HCC mice was significantly lower than those in control or NAFLD mice, and was negatively correlated with the degree of hepatocyte malignant transformation[90,91]. A series of experiments confirmed that CPT-II was inactivated, suggesting that low CPT-II expression in IMM might lead to liver lipid accumulation and participate in promoting NAFLD malignant transformation[92].

CPT-II INVERSE-CORRELATED WITH HCC MARKERS

Based on clinical and basic evidences, abnormal lipid accumulation was associated with NAFLD malignant transformation. However, only a few studies have been reported on the relationship between CPT-II activation and HCC progression. The alteration of CPT-II expression might be an important link

in the obstruction of FAO and abnormal lipid accumulation[93]. Based on above findings, some scholars sequenced the whole gene of the mitochondrial CPT-II in NAFLD patients, and found that *CPT2* variation was significantly associated with CPT-II activity that might be the key factor of NAFLD or related cirrhosis/HCC because its inactivation is closely related to an energy production disorder in models of NAFLD[94,95]. The dynamic alterations of CPT-II expression located on IMM during malignant transformation of hepatocytes in SD rats induced by chemical carcinogens (2-fluorenylacetamide, 2-FAA) were investigated under lipid accumulation[19]. For the first time, the progressively decreasing expression of CPT-II at mRNA or protein level were reported, and the significantly increasing HCC related to molecular markers were confirmed during the rat hepatocarcinogenesis.

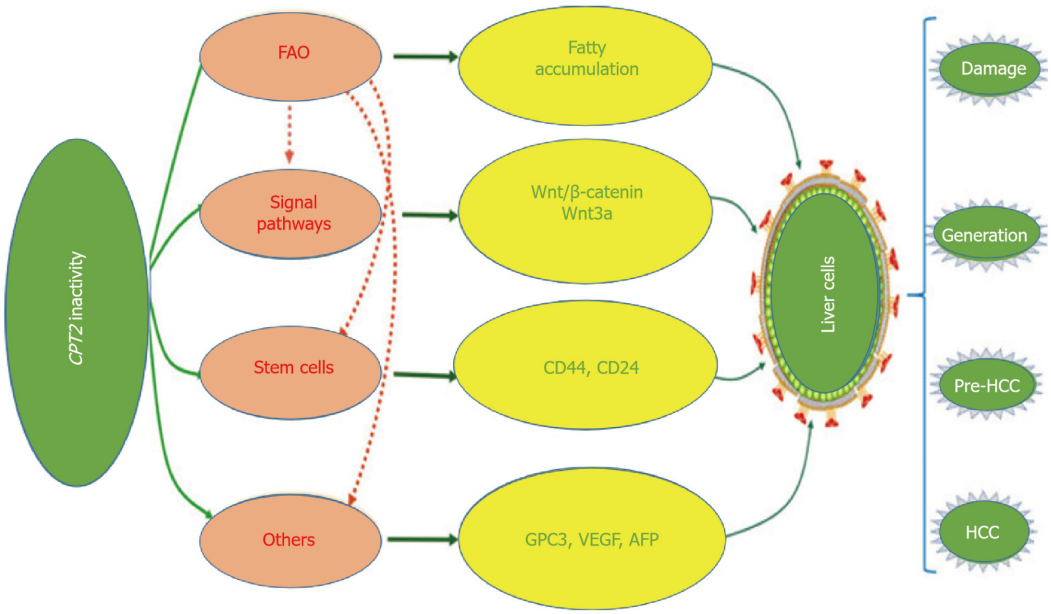
There has been a rise in the prevalence of NAFLD, paralleling a worldwide increase in MAFLD and HCC[96]. According to the dynamic pathological alterations of the model, a continuum of morphological abnormalities on liver sections has a variable course, from normal hepatocytes, lipid accumulation, cell denaturation, precancerous lesion and HCC formation. Compared with the control liver sections, there was a large amount of fat in the hepatocytes of the model rats by the oil red O staining; In the meantime, transmembrane glycoprotein CD44 activation promotes inflammatory cell recruitment and plays a key role of being linked to NAFLD progression to HCC[97-99]. CPT-II has been demonstrated to interact in forming supramolecular complexes that facilitate the passage of acylcarnitine and its expression was gradually decreased in malignant trans-formation of hepatocytes in NAFLD. However, the reported HCC biomarkers such as AFP, GPC3[100] and Wnt3a[101] were significantly increasing expressions in hepatocarcinogenesis except for CD44 as one of the most frequently reported cancer stem-like cell markers[102]. These data suggested that the alteration of CPT-II expression should be associated with the malignant progression of NAFLD.

Metabolically related NAFLD is emerging as a major cause of HCC in Western countries[103-105]. This presents an additional challenge, as NAFLD-related HCC tend to be advanced in elderly patients with comorbidities and their prognosis is very poor[106]. The pathogenesis of NAFLD-associated HCC is multifactorial and remains to be identified, although the risk of hepatocarcinogenesis is undoubtedly increased as NAFLD progresses to NASH and cirrhosis[107]. The new findings of CPT-II are useful for understanding NAFLD and HCC and should hopefully lead to the development of clinically relevant biomarkers and strategies to help identify high-risk patients, early use of preventive measures or better treatment[108]. Energy metabolism is a prerequisite for maintaining normal life activities. NAFLD is caused by excessive lipid accumulation in hepatocytes and is regarded as one of the most common liver diseases[109,110]. The down-regulation of *CPT2* in IMM was one of the main causes of acyl carnitine accumulation, which was also seen in malignant transformation of hepatocytes, suggesting that CPT-II inactivity or dysfunction might become a new mechanism of blocked lipid oxidation for HCC.

CONCLUSION

Owing to its high prevalence and potential risk, NAFLD has become a major health concern worldwide. Hepatocyte CPT-II variation or activity alteration undoubtedly has a significant impact on aggravating liver fatty accumulation, inducing activation of cancer-related stem cells, and malignant transformation of hepatocytes (Figure 2), especially in patients with HBV chronic infection. The phenomenon of CPT-II inactivation as warning signs of NAFLD malignant transformation needs attention. However, its specific regulatory mechanism is unknown, so this is a good research prospect. At present, the exact relationship between CPT-II and NAFLD remains to be explored. It is believed that with the vigorous development of molecular biological theory and technology, understanding the function of CPT-II physiology will continue to deepen, which has guided significant knowledge for CPT-II alteration during NAFLD progression. At the same time, it also brings hope and provides a theoretical basis for the assumption of early intervention of NAFLD or human related diseases and CPT-II can be used as a molecular target for monitoring or therapy.

Hepatocyte CPT-II variation or activity alteration undoubtedly has a significant impact on aggravating liver fatty accumulation, inducing activation of cancer-related stem cells, and malignant transformation of hepatocytes.



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Figure 2 Carnitine palmitoyltransferase-II inactivity in hepatocarcinogenesis. Disorder of lipid metabolism may be related to the pathogenesis of nonalcoholic fatty liver disease with malignant transformation of hepatocytes. AFP: Alpha-fetoprotein; CoA: Coenzyme A; CPT2: Carnitine palmitoyl transferase 2; FAO: Fatty acid oxidation; HCC: Hepatocellular carcinoma; VEGF: Vascular endothelial-derived growth factor.

FOOTNOTES

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REVIEW

Obesity and novel management of inflammatory bowel disease

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Abstract

Obesity is prevalent within the inflammatory bowel disease (IBD) population, particularly in newly developed countries. Several epidemiological studies have suggested that 15%-40% of IBD patients are obese, and there is a potential role of obesity in the pathogenesis of IBD. The dysfunction of mesenteric fat worsens the inflammatory course of Crohn's disease and may induce formation of strictures or fistulas. Furthermore, obesity may affect the disease course or treatment response of IBD. Given the increasing data supporting the pathophysiologic and epidemiologic relationship between obesity and IBD, obesity control is being suggested as a novel management for IBD. Therefore, this review aimed to describe the influence of obesity on the outcomes of IBD treatment and to present the current status of pharmacologic or surgical anti-obesity treatments in IBD patients.

Key Words: Obesity; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Core Tip: Obesity is prevalent within the inflammatory bowel disease (IBD) population, particularly in newly developed countries. The dysfunction of mesenteric fat worsens the inflammatory course of Crohn's disease and may induce formation of strictures or fistulas. Furthermore, obesity may affect the disease course or treatment response of IBD. Along with the increasing data that support pathophysiologic and epidemiologic relationship between obesity and IBD, attention is being focused on obesity control as a novel management of IBD. The main purpose of this review is to describe the influence of obesity on the outcomes of IBD treatment and to present the current status of pharmacologic or surgical anti-obesity treatments in IBD patients.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory condition with an unclear etiology and pathophysiology that remain to be fully elucidated[1,2]. Obesity is a pathological condition in which there is an abnormal or excessive accumulation of body fat resulting from an imbalance between energy intake and consumption. In Western populations, a body mass index (BMI) exceeding 25 kg/m^2 is commonly classified as an overweight condition, and a BMI over 30 kg/m^2 is regarded as an obese condition. In Asian populations, the diagnostic thresholds for obesity and overweight have been established as BMIs of 25 kg/m^2 and 23 kg/m^2, respectively[3]. Obesity is a clear risk factor for a spectrum of chronic diseases, including type 2 diabetes, cardiovascular disease, respiratory problems, and cancer. Moreover, obesity is also associated with the occurrence of autoimmune diseases such as rheumatoid arthritis, psoriasis, and systemic lupus erythematosus[4].

The worldwide prevalence of obesity has nearly tripled since 1975[5], and IBD has also shown a similar trend[6]. This analogous increase is perhaps related to lifestyle changes caused by westernization and urbanization, including the lack of exercise and a westernized diet, which are common risk factors for obesity and IBD. Historically, it has been common for clinicians to associate IBD patients with a low or normal BMI due to the complications of IBD such as decreased food intake, malabsorption, weight loss, and nutritional deficiencies. However, obesity is increasingly being associated with IBD due to its overall pro-inflammatory effect. Several epidemiological studies have suggested that 15%-40% of IBD patients are obese[7-11], and hypothesize that obesity contributes to the development of IBD. Furthermore, obesity may affect the disease course or treatment response of IBD. With the increasing data supporting pathophysiologic and epidemiologic relationship between obesity and IBD, research interest on interventions for obesity as a novel management of IBD is also increasing.

The present review aims to provide a comprehensive summary of the pathophysiology of obesity in IBD, the influence of obesity on IBD outcomes, the effect of obesity on IBD management, and the potential impact of obesity treatment on IBD outcomes. Further, this review aimed to present the current status of anti-obesity treatments in IBD patients.

PREVALENCE OF OBESITY IN IBD PATIENTS

Previous studies have reported that 15%-40% of IBD patients are obese and 20%-40% are overweight[7-11]. Additionally, severe obesity (BMI ≥ 40 kg/m^2) is also reported in 2%-3.2% of IBD patients[7]. The prevalence of obesity among IBD patients has been observed to increase over time, which appears to correspond with the global trend of rising obesity rates. A single-center study in France confirmed that the proportion of obesity among CD patients from 1974 to 2000 increased from 1.7% before 1981 to 4% after 1990[12]. A single-center study in Korea, utilizing data from the Asan IBD Registry from 1989 to 2016, reported that out of 6803 patients diagnosed with IBD, 16 with CD and 27 with UC were classified as obese (BMI ≥ 30 kg/m^2). The study did not reveal any clinically meaningful differences between the obese and non-obese patient groups[13]. An analysis of 10282 CD patients enrolled in a randomized controlled clinical trial revealed a significant increase in mean BMI at enrollment, which rose from 20.8 kg/m^2 in 1991 to 27.0 kg/m^2 in 2008[14].

RELATIONSHIP BETWEEN OBESITY AND IBD

Obesity as a precursor to the onset of IBD

Obesity may be a potential risk factor for developing IBD, particularly CD (Table 1). In the Unite States Nurses' Health Study, which followed around 110000 women, found that obesity at age 18 was a significant predictor for the development of CD but not UC, when compared to individuals with a normal BMI [adjusted hazard ratio (aHR) = 2.33; 95% confidence interval (CI): 1.15-4.69][15]. This study exhibited that a higher degree of weight gain (between the ages of 18 and enrolment) was linked with elevated risk of developing CD (weight gain > 13.6 kg *vs* < 2.3 kg; HR = 1.52; 95%CI: 0.87-2.65). The Copenhagen School Health Records Register cohort study investigated the potential association between BMI during the ages of 7 to 13 years and the onset of adult-onset IBD[16]. Obesity in early adolescence has been shown to increase the risk of CD before the age of 30 years (HR = 1.2; 95%CI: 1.1-1.3) while decreasing the risk of UC (HR = 0.9; 95%CI: 0.9-1.0). In a recent meta-analysis of five prospective cohort studies, obesity was found to be associated with an elevated risk of developing older-onset CD, while no significant association was found with UC[17]. The analysis showed that obese patients had an increased risk of developing CD compared to those with a normal BMI (aHR = 1.34; 95%CI: 1.05-1.71; I^2 = 0%). Moreover, every 5 kg/m^2 increase in baseline BMI was found to correspond to a 16% increase in the risk of CD (aHR = 1.16; 95%CI: 1.05-1.22; I^2 = 0%). However, the European Prospective Investigation into Cancer and Nutrition-IBD study, which involved a large sample size, concluded no significant correlation between BMI and the incidence of IBD, showing contrasting results to previous studies[18].

Obesity caused by IBD

Weight gain may occur during the treatment of IBD. Several preclinical data suggest that, in IBD patients, imbalance of gut microbiota and altered metabolic intestinal signaling mediated by hormones, bile acids, and satiety-related peptides have been implicated in the development of obesity and dysmetabolism[19,20]. Smoking cessation as a lifestyle modification and utilization of corticosteroids could affect the weight gain in IBD patients[21,22]. Additionally, there are a few studies investigating weight gain among IBD patients treated with biologic therapies, especially anti-tumor necrosis factor alpha (TNF)-α therapy. In 21 CD patients, total abdominal fat increased by 18% after 8 wk of infliximab induction therapy (P = 0.027)[23]. A recent retrospective study examined the longitudinal changes in weight among patients with IBD receiving different biologic drugs including infliximab, adalimumab, vedolizumab and ustekinumab[24]. The study revealed a statistically significant increase in body weight over time among patients receiving infliximab and vedolizumab, with the infliximab/vedolizumab group experiencing a greater degree of weight gain as compared to the adalimumab group. Moreover, observed weight gain among IBD patients undergoing biologic therapy was found to be linked with other clinical factors, such as male gender, high levels of C-reactive protein (CRP), and low serum albumin.

OBESITY AND IBD PATHOGENESIS

Obesity induces a chronic, low-grade inflammatory state, characterized by the release of multiple pro-inflammatory signaling molecules from hypertrophic adipocytes in mesenteric visceral adipose tissue (VAT)[25-27]. Mesenteric VAT is characterized by the presence of M1 macrophages that secrete a variety of inflammatory cytokines and it has the potential to affect intestinal barrier function[28,29]. Several studies have established a relationship between adipocyte mass and the degree of cytokine expression [30]. Obesity has been shown to be positively associated with visceral adiposity, as determined by volumetric cross-sectional imaging analysis. Furthermore, in obese individuals, there is a direct correlation between visceral adiposity and circulating levels of interleukin-6 (IL-6). Additionally, BMI has been found to be linked with increased levels of CRP in this population[31].

Other mechanisms of obesity in IBD include intestinal barrier dysfunction and alterations in the intestinal microbiota (Figure 1). Obesity and IBD are associated with dysbiosis along with a reduction in bacterial diversity[32]. High fat diet during obesity decreases the diversity of the gut microbiota (dysbiosis), which, in turn, leads to the reduction of anti-inflammatory bacterial species or metabolites. These changes can trigger innate immune system by activation of pattern recognition receptors, including Nod-like receptors or Toll-like receptors, which are present on intestinal epithelial cells or dendritic cells. Pro-inflammatory cytokines secreted from innate immune system can also contribute to increased intestinal permeability, which may increase the risk of bacterial translocation[17,25]. Numerous studies have demonstrated that impaired gut barrier function is a characteristic feature of obesity. This dysfunction permit increased bacterial translocation, leading to the subsequent initiation of systemic inflammation[33,34].

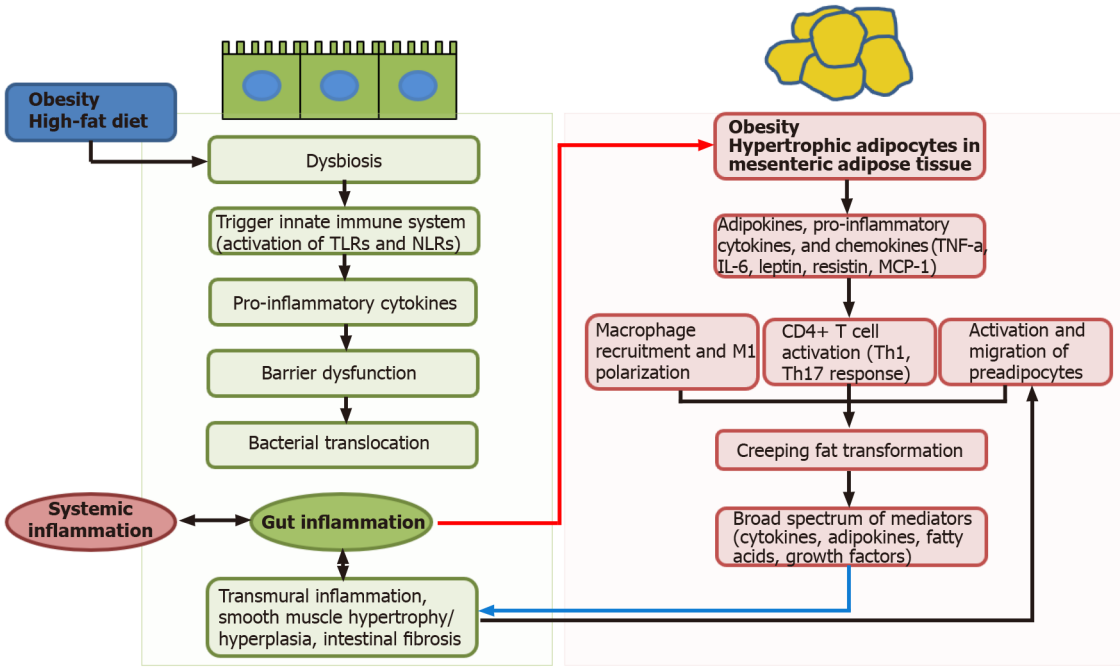
Accumulating evidence suggests a connection between changes in the mesenteric fat and IBD, in particular CD[27]. Intestinal barrier dysfunction and transmural inflammation may induce bacterial translocation to the surrounding mesenteric adipose tissue, which subsequently leads to the adipocyte

Table 1 Relationship between obesity and inflammatory bowel disease (obesity as a precursor to the onset of inflammatory bowel disease)

Ref.	Study design and study population	Key findings
Khalili *et al*[15], 2015	United States Nurses' Health Study cohort study: Prospective cohort study of United States women (n = 111498 women); BMI at age 18, baseline, and every 2 yr since baseline was obtained; 2028769 person-years of follow up. CD (n = 153); UC (n = 229)	Obesity at age 18 was an independent risk factor for the development of CD compared to normal BMI (aHR = 2.33, 95%CI: 1.15-4.69). No association between BMI at age 18, baseline BMI, and updated BMI and risk of UC. Higher weight gain was associated with increased risk of CD (P_{trend} = 0.04). A greater magnitude of weight gain (from age 18 to age at enrolment) associated with increased risk of developing CD (weight gain > 13.6 kg *vs* < 2.3 kg, HR = 1.52, 95%CI: 0.87-2.65). No association between weight change (from age 18 to baseline) and risk of UC (Pt_{rend} = 0.17) (weight gain > 13.6 kg *vs* < 2.3 kg, HR = 0.92, 95%CI: 0.60-1.40)
Harpsøe *et al*[101], 2014	Danish National Birth Cohort study: A large population-based cohort study (n = 75008 women); BMI: Obtained at study baseline (based on prepregnancy body weigh); median 11.4 yr of follow-up. CD (n = 138); UC (n = 394)	An increased risk of developing fetal CD in both underweight (HR = 2.57, 95%CI: 1.30-5.06) and obese women (HR = 1.88, 95%CI: 1.02-3.47) compared with normal-weight women, pointing to a U-shaped association. No association between pregnancy obesity and risk of developing UC (HR = 0.77, 95%CI: 0.48-1.25)
Jensen *et al*[16], 2018	Copenhagen School Health Records Register cohort study: Cohort from the Copenhagen School Health Records Register (n = 316799); relationship between BMI in the ages of 7 to 13 yr and adult-onset IBD; BMI: Obtained at ages 7 through 13 yr; approximately 10 million person-years of follow-up. CD (n = 1500); UC (n = 2732)	Obesity in early adolescence (at each age from 7 to 13 yr) increased the risk of CD diagnosed before age 30 yr (HR = 1.2, 95%CI: 1.1-1.3) while decreasing the risk of UC (HR = 0.9, 95%CI: 0.9-1.0). No associations between changes in BMI between 7 and 13 yr and later risk of CD or UC
Chan *et al* [17], 2022	Pooled analysis of 5 prospective cohort studies from the Dietary and Environmental Factors IN-IBD study (n = 601009): BMI: Obtained at study baseline and during follow-up period; 10110018 person-years of follow-up. CD (n = 563); UC (n = 1047)	Obesity was associated with an increased risk of older-onset CD but not UC. The risk of developing CD increased in obese patients compared against those with a normal BMI (aHR = 1.34, 95%CI: 1.05-1.7, I^2 = 0%). Each 5 kg/m^2 increment in baseline BMI was associated with a 16% increase in risk of CD (aHR = 1.16, 95%CI: 1.05-1.22; I^2 = 0%). With each 5 kg/m^2 increment in early adulthood BMI (age 18-20 years), there was a 22% increase in risk of CD (pooled aHR = 1.22, 95%CI: 1.05-1.40, I^2 = 13.6%). An increase in waist-hip ratio was associated with an increased risk of CD that did not reach statistical significance (pooled aHR across quartiles = 1.08, 95%CI: 0.97-1.19, I^2 = 0%). No associations were observed between measures of obesity and risk of UC. For every 5 kg/m^2 increase in BMI, the multivariable-adjusted HR was 1.00 (95%CI: 0.90-1.05). For every 5 kg/m^2 increase in early adulthood BMI, the multivariable-aHR for UC was 1.05 (95%CI: 0.90-1.22, I^2 = 0%)
Chan *et al* [18], 2013	European Prospective Investigation into Cancer and Nutrition-IBD study (n = 300724): BMI: Obtained at study baseline and during follow-up period. CD (n = 75); UC (n = 177)	No associations with the four higher categories of BMI compared with a normal BMI for UC (P_{trend} = 0.36) or CD (P_{trend} = 0.83). The lack of associations was consistent when BMI was analyzed as a continuous or binary variable (BMI 18.5 < 25.0 *vs* ≥ 25 kg/m^2). Physical activity and total energy intake, factors that influence BMI, did not show any association with UC (physical activity, P_{trend} = 0.79; total energy intake, P_{trend} = 0.18) or CD (physical activity, P_{trend} = 0.42; total energy, P_{trend} = 0.11)

BMI: Body mass index; IBD: Inflammatory bowel disease; aHR: Adjusted hazard ratio; CI: Confidence interval; CD: Crohn's disease; US: Ulcerative colitis.

hypertrophy (red arrow, Figure 1). The hypertrophic adipocytes releases pro-inflammatory cytokines, adipokines, and chemokines such as TNF-α, IL-6, leptin, resistin, and monocyte chemoattractant protein-1). These pro-inflammatory mediators can induce the infiltration of M1 macrophages and CD4+ T cell activation into T helper (Th) 1 and Th17 cells[35]. This process may contribute to the development of hypertrophic mesenteric fat wrapping around the inflamed intestine, known as "creeping fat" that is pathognomonic of CD[27]. Creeping fat itself is thought to be an intrinsic component of inflammatory dysregulation in the pathogenesis of CD. Compared to other VAT, creeping fat demonstrates greater immunological activity. Additionally, there is a significant correlation between the extent of creeping fat and the degree of histological inflammation, as well as the level of infiltration by lymphocytes or macrophages[36]. In addition, the creeping fat formation may be induced by migration of preadipocytes from mesenteric VAT in response to the increased fibronectin signal released from activated muscularis propria smooth muscle cells in inflamed gut[37]. The presence of creeping fat in CD patients has been associated with various pathological changes, including muscularis propria hyperplasia, transmural inflammation, and intestinal fibrosis. These alterations may contribute to the stricturing form of the disease[37,38]. Notably, creeping fat has been found to contain higher levels of fibrotic tissue and T cells in the ileum, as compared to colonic fat from patients with either CD or UC[39]. Creeping fat secretes a broad spectrum of pro-inflammatory and pro-fibrotic mediators such as adipokines, cytokines, fatty acids, and growth factors[40]. Moreover, expression of leptin and adiponectin is elevated in the creeping fat of CD patients. The severity and activity of CD have been correlate with the levels of other adipokines, including resistin[41]. A case-control study showed that the gene expression profile of creeping fat in CD patients was similar to that of VAT from obese patients, indicating a greater inflammatory state as compared to VAT obtained from non-obese individuals[42].



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Figure 1 High fat or Western diets decreases the diversity of the gut microbiota (dysbiosis), leading to the reduction of anti-inflammatory bacterial species or metabolites. These changes can trigger innate immune system by activation of pattern recognition receptors such as Toll-like receptors or Nod-like receptors on intestinal epithelial cells or innate immune cells. Pro-inflammatory cytokines secreted from innate immune system induces barrier dysfunction and subsequent bacterial translocation. Low grade chronic inflammation in gut and bacterial translocation to mesenteric adipose tissue leads to the adipocyte hypertrophy (red arrow). The hypertrophic adipocytes release adipokines, pro-inflammatory cytokines, and chemokines such as tumor necrosis factor alpha, interleukin-6, leptin, resistin, and monocyte chemoattractant protein-1. These pro-inflammatory mediators can induce macrophage recruitment and polarization and CD4+ T cell activation into T helper 1 (Th1) and Th17 cells. Finally, this process can drive the hypertrophic mesenteric fat wrapping around the inflamed bowel, creeping fat. Creeping fat secretes a broad spectrum of mediators, and induce smooth muscle hypertrophy/hyperplasia, transmural inflammation, intestinal fibrosis. TLR: Toll-like receptor; NLR: Nod-like receptor; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin; MCP-1: Monocyte chemoattractant protein 1; Th: T helper.

IMPACT OF OBESITY ON THE NATURAL HISTORY AND OUTCOMES OF IBD

The effect of obesity on the disease phenotype of IBD remains uncertain. While a retrospective review has indicated that obesity may be a risk factor for perineal disease in IBD[12], other studies have not found any differences in disease distribution or behavior between patients with UC or CD who are obese compared to those who are not[8,43]. Obesity has been linked to increased disease activity and unfavorable clinical outcomes in several chronic inflammatory diseases, such as psoriasis and rheumatoid arthritis[44,45]. However, evidence regarding the effect of obesity on outcomes in IBD is limited and inconclusive. A study of 581 IBD patients showed that obese individuals had significantly lower frequencies of hospitalization (42.1% *vs* 66.0%, $P < 0.001$) and a reduced likelihood of requiring surgical intervention (41.1% *vs* 61.1%, $P = 0.02$), when compared to those patients with normal BMI[8]. However, in other population-based study for 143190 IBD patients, it was demonstrated that obesity was an independent predictor of higher rates of all-cause re-admission at both 30 d [18% *vs* 13%; adjusted odds ratio (aOR) = 1.16; $P = 0.005$] and 90 d (29% *vs* 21%; aOR = 1.27; $P < 0.0001$), in comparison with non-obese patients[46]. A systematic review reported that there is no significant difference in the rates of corticosteroid usage, hospitalization, surgical intervention, or emergency room visits between obese and normal-weight individuals with IBD[7]. This finding has been supported by a recent retrospective study conducted in South Korea, which found no significant differences in clinical features or treatment outcomes between obese and non-obese patients with IBD, as measured by the cumulative probabilities of receiving various treatments[13].

In CD, many studies show that obesity may indeed have a beneficial effect. A previous report supports this hypothesis by showing that the small VAT adipocytes in CD patients exhibit higher anti-inflammatory genes, which implies a potential protective role of VAT in CD[42]. A retrospective analysis of 221 patients with CD found that an increase of 1 kg/m^2 in BMI at diagnosis led to a 5% reduction in the risk of requiring surgical intervention in the future (HR = 0.95; 95%CI: 0.91-0.99). Whereas, the risk of corticosteroid use or future hospitalization did not differ[47]. The results of a retrospective study showed that obese patients with CD had lower frequencies of corticosteroid usage, anti-TNF treatment, hospitalization, and surgery in contrast to those with normal weight[8]. However, there were also contrasting results. For instance, one study reported that obese patients with CD were more at risk of active disease (OR = 1.50; 95%CI: 1.07-2.11) or hospitalization (OR = 2.35; 95%CI: 1.56-

3.52)[12]. Alternatively, a prospective case-control study revealed no correlation between BMI and the risk of undergoing surgery or using corticosteroids in the future[9].

In UC, several studies suggest that obesity may have a worse prognosis. In one population-based cohort study that included 267 patients with UC, each incremental increase in BMI by 1 kg/m^2 was associated with a 3.4% increase in the risk of hospitalization (P = 0.052) and a 6% increase in the risk of surgery (P = 0.01)[47]. A recent population-based cohort of 417 UC patients, of whom 20.6% were obese, confirmed that obesity among individuals with UC may have unfavorable prognostic implication, particularly with regards to the subsequent hospitalization (HR = 1.72; 95%CI: 1.10-2.71; P = 0.018) and corticosteroid usage (HR = 1.026; 95%CI: 1.00-1.05; P = 0.05) in comparison to patients with normal weight[48]. Moreover, an increase of 1 kg/m^2 in BMI was associated with a 5% escalation in the probability of subsequent hospitalization (HR = 1.05; 95%CI: 1.01-1.08; P = 0.008) and a 2.6% increase in the chance of requiring corticosteroid usage (HR = 1.026; 95%CI: 1.00-1.05; P = 0.05). However, the retrospective analysis of 284 UC patients showed that obese people displayed a reduced risk of developing complications[8].

IMPACT OF OBESITY ON THE MANAGEMENT OF IBD

Medical management

There is evidence to suggest that obesity may be an adverse prognostic factor in a patient's response to drug therapy. The reason being obesity is considered a chronic inflammatory state and obesity can affect the pharmacokinetics of drugs. Obesity may decrease drug half-life and trough levels due to an increase in drug clearance and volume of distribution.

Immunomodulators: Despite extensive clinical experience with thiopurine, few studies have evaluated clinical predictors of response to thiopurine in IBD. A retrospective study of 1176 IBD patients evaluating azathioprine responsiveness based on BMI found that a reciprocal association between BMI and outcomes in patients with UC and CD. More specifically, favorable outcomes were observed in UC patients with a BMI below 25 kg/m^2 and in CD patients with a BMI exceeding 25 kg/m^2[49]. In a cohort of 132 patients with IBD treated with thiopurines dosed according to BMI, a study found that patients with obesity were less likely to reaching optimal therapeutic concentrations of the thiopurine metabolite, 6-thioguanine, which has been shown to correlate with remission of IBD[50].

Anti TNF-α therapy: Several data have suggested that obesity may induce a reduced response to anti-TNF-α agents[51]. The augmented clearance and expanded volumes of distribution associated with obesity can lead to lower trough levels, resulting in either a diminished response or a heightened risk of response loss to anti-TNF-α agents. For adalimumab, body weight has been shown to be the most significant predictor of drug clearance and volume of distribution in psoriasis patients[52]. In contrast to other rheumatoid conditions, studies examining the impact of obesity on anti-TNF therapy in IBD have produced inconsistent findings.

Obesity has been linked to an increased risk of failure of anti-TNF therapy, irrespective of the method of administration (subcutaneous *vs* intravenous) and dosing regimens (weight-based *vs* fixed-dose regimens). These results could imply that there is an intrinsic factor to obesity that reduces treatment response, regardless of drug level and mode of exposure. A recent meta-analysis found that obese patients with UC were at a greater risk of anti-TNF-α treatment failure (OR = 1.41; 95%CI: 1.01-1.98; P = 0.045) than non-obese patients, irrespective of whether they received fixed-dose or weight-based treatment regimens[53]. Furthermore, in another study that evaluated 160 UC patients treated with various biologic agents, both weight-based (infliximab) and fixed-dose regimens (adalimumab, certolizumab, vedolizumab, and golimumab), regardless of the type of biologic used, an increase in BMI of 1 was associated with a 4% greater risk of treatment failure (aHR = 1.04; 95%CI: 1.00-1.08) for all patients [54]. However, a pooled data analysis of over 1200 infliximab-treated IBD patients reported that BMI did not have a significant effect on clinical remission rate[55]. A metaanalysis for multiple immune-mediated diseases showed that obesity was associated with anti-TNF therapy failure in several rheumatic diseases, but no such association was observed in IBD[56]. However, in a recent meta-analysis including the newer 6 articles into the meta-analysis, obesity was associated with higher risk of failing anti-TNF therapy in UC patients, but not in CD patients[53]. Although there are insufficient studies on the impact of obesity on safety of biologic agents, a cohort study of over 6000 biologic-treated IBD patients reported no association between obesity and an increased risk of serious infections[57].

Vedolizumab: There is insufficient data regarding the potential impact of obesity on the therapeutic efficacy of vedolizumab in the management of IBD. Vedolizumab trough drug levels are known to be inversely proportional to body weight[58]. A recent retrospective study showed that obesity was not significantly related to increased rates of dose escalation for vedolizumab therapy[59]. However, a higher BMI was linked to reduced rates of vedolizumab discontinuation and CRP normalization, although not with endoscopic remission.

Ustekinumab: In chronic inflammatory conditions other than IBD, such as psoriasis and rheumatoid arthritis, obesity may negatively affect the clinical response to biological drugs. Of note, anti-interleukin drugs appear to be more strongly affected by BMI than anti-TNF-α drugs. However, in IBD, there is very little research on the impact of obesity on treatment response with this drug. A *post hoc* analysis of IM-UNITI study, which evaluated the efficacy of ustekinumab for maintenance of CD, revealed a statistically significant decrease in ustekinumab trough levels among obese patients (median 2.98 mcg/mL) compared to patients with overweight (4.84 mcg/mL; $P = 0.021$) and underweight or normal weight (4.43 mcg/mL; $P = 0.014$)[60]. While BMI appeared to have an impact on ustekinumab drug levels, it was not found to be a significant predictor of clinical remission.

Tofacitinib: A pharmacokinetic profile of tofacitinib revealed that increased body weight had some effect on the drug's plasma concentrations, with higher body weight associated with lower peak and higher trough concentrations. However, these differences were considered to be not clinically significant [61]. The evidence suggests that psoriatic arthritis patients with a baseline BMI over 35 kg/m^2 have lower rates of response to tofacitinib than those with lower baseline BMI[62]. However, in a *post hoc* analysis of the OCTAVE studies, effectiveness and safety profile of tofacitinib in UC patients is not impacted by obesity[63]. In the UC population, BMI was not identified as a significant predictor of outcomes, and no clear relationship between BMI and adverse events was observed.

Surgical management

It has been well established that obesity increase operative time and the risk for conversion to laparotomy[64]. Obesity also may heighten the risk of short-term perioperative complications, such as surgical site infections and wound complications after abdominal surgery[65]. Obesity also makes surgery for IBD more challenging, particularly those requiring pelvic exposure. A study involving 382637 inpatient hospitalizations for surgery in IBD patients revealed that obese patients had significantly higher rates of postoperative complications. Specifically, the study found that obese patients had increased odds of postoperative wound complications (OR = 1.35, $P = 0.01$), pulmonary complications (OR = 1.21, $P = 0.02$), infections (OR = 1.16, $P = 0.02$), and shock (OR = 1.30, $P = 0.02$)[66]. A recent meta-analysis strongly supported the association between obesity and postoperative complications in IBD[67].

Stoma creation and ileal pouch-anal anastomosis (IPAA) in obese patients are more difficult to perform and also appear to increase the risk of complications in IBD patients. Obesity has been identified as a risk factor for stoma-related complications[68]. Additionally, it may increase the likelihood of postoperative complications, including pelvic sepsis, which has been shown to have a negative impact on long-term pouch function[69]. However, several studies have indicated that overweight and obese patients who undergo a three-stage IPAA procedure with the utilization of a diverting stoma may achieve comparable long-term outcomes to non-obese patients[64].

IMPACT OF OBESITY TREATMENT ON IBD OUTCOMES

Given the rising prevalence of obesity within the IBD population, there is a growing need to explore the effects of weight loss interventions on outcomes of IBD. It is widely recognized that weight loss can have a favorable influences on the outcome of numerous chronic diseases[70]. Despite considerable evidence that obesity has a negative influence on the response to therapies for IBD, there is currently a lack of data regarding whether interventions aimed at treating obesity can improve outcomes in individuals with IBD. To date, no interventional studies have been conducted specifically to investigate the impact of intentional weight loss on IBD.

Current therapeutic strategies to obesity includes lifestyle modifications, pharmacologic treatment, bariatric surgery, and bariatric endoscopic applications, of which the intragastric balloon is the most widely used. The European Society for Clinical Nutrition and Metabolism (ESPEN)/United European Gastroenterology (UEG) guidelines suggest that the management of obesity in patients with IBD should involve a stepwise approach, starting with dietary and lifestyle interventions. If necessary, anti-obesity drugs or bariatric surgery can be considered as options (Table 2). However, the guidelines do not provide a one-size-fits-all approach and individualized care is recommended[71].

Lifestyle and dietary interventions

A combination of dietary adjustments, physical exercise, and behavioral modifications as part of a lifestyle intervention is generally considered the preferred approach for achieving weight loss in the general population. Of course, IBD patients should also consider this approach, but there are special considerations in this cohort that require the advice of a certified dietitian. Specifically, individuals with active IBD may find it challenging to tolerate certain "healthy" foods, such as fruits and vegetables, which are commonly recommended as part of a healthy diet. Additionally, IBD patients, despite being overweight, may be vulnerable to the deficiency of various vitamins and micronutrients, and thus

Table 2 Impact of obesity treatment on inflammatory bowel disease outcomes

Interventions		Study design	Key finding	Ref.
Lifestyle and dietary interventions	Diet: No data on the effects of overall calorie intake or supervised dietary weight loss on outcomes in IBD patients	Retrospective study: (1) Impact of mediterranean diet on the liver steatosis, clinical disease activity, and QoL in IBD patients (n = 142); (2) 84 UC, 58 CD; and (3) BMI: Collected at study baseline and after 6 mo	Diet-adherent CD and UC improved BMI (UC: -0.42, P = 0.002; CD: -0.48, P = 0.032) and waist circumference (UC: -1.25 cm, P = 0.037; CD: -1.37 cm, P = 0.041). The number of patients affected by liver steatosis of any grade was significantly reduced in both groups after mediterranean diet intervention (UC: 36.9% *vs* 21.4%, P = 0.0016; CD: 46.6% *vs* 31.0%, P < 0.001). Mediterranean diet improved QoL in both UC and CD	Chiccoet al[72], 2021
	Exercise: (1) Anti-inflammatory effects through a variety of mechanisms, including reducing visceral fat, reducing the secretion of inflammatory adipokines, and reducing stress-induced intestinal barrier dysfunction; and (2) Experts have recommended a prescription of exercise for IBD patients that consists of walking 20-30 min at 60% of maximal heart rate 3 d per week along with resistance training 2-3 times per week for its impact on bone mineral density[102], however this has not been tested prospectively	Prospective study: IBD patients with mild active disease or in remission (n = 32)	IBD patients performed low-intensity walking at an interval of 3 times per week for a duration of 3 mo. IBD patients who exercise have improved sense of well-being and QoL	Ng *et al* [103], 2007
		30 patients with moderate-to-mild CD. Randomized to moderate-intensity running 3 × weekly for 10 wk *vs* usual care	No significant difference in total IBDQ scores, IBDQ social subscores did improve in intervention group (P = 0.023). No disease exacerbation	Klare *et al* [104], 2015
		Prospective study: Using the Crohn's and Colitis Foundation of America Partners Internet-based cohort of IBD patients (n = 1857); 549 UC, 1308 CD	Reduced risk of CD exacerbation (RR = 0.72, 95%CI: 0.55-0.94), reduced risk of UC exacerbation (RR = 0.78, 95%CI: 0.54-1.13), with higher levels of exercise	Jones *et al* [74], 2015
Pharmacologic treatment: BMI of 30 kg/m^2 or a BMI of 27 kg/m^2 with obesity-related diseases (*e.g.*, hypertension, type 2 diabetes mellitus, and sleep apnea)	Orlistat: (1) By inhibiting gastric and pancreatic lipases, reducing absorption of monoaclglycerides and free fatty acids; and (2) Should be avoided in IBD patients because of the mechanism of action and common side effect	No data on the effect of Orlistat on outcomes in IBD patients		
	Liraglutide: Glucagon-like peptide-1 receptor agonist also known as incretin mimetics	Case report: CD patient with type 2 diabetes and active CD	Switching from insulin to liraglutide improved glycemic control and the QoL scores	Kuwata *et al*[76], 2014
		A nationwide cohort study using Danish registries: Patients with IBD and type 2 diabetes (n = 3751)	A lower risk of adverse clinical events (a composite of the need for oral corticosteroid treatment, need for TNF-α-inhibitor treatment, IBD-related hospitalization, or IBD-related major surgery) amongst patients treated with GLP-1 based therapies compared with treatment with other antidiabetic therapies (adjusted IRR = 0.52, 95%CI: 0.42-0.65)	Villumsen *et al*[77], 2021
	Naltrexone/bupropion: Naltrexone and bupropion alone may have anti-inflammatory properties	Uncontrolled studies of IBD patients not in remission (n = 47): Low-dose naltrexone for 12 wk	Low dose naltrexone induced clinical improvement in 74.5%, and remission in 25.5% of patients	Lie *et al* [78], 2018
		Retrospective study of IBD patients who had received low-dose naltrexone (n = 582)	Initiation of low-dose naltrexone in IBD was followed by reduced dispensing of several drugs considered essential in the treatment of IBD	Raknes *et al*[79], 2018
	Phentermine/topiramate: (1) A highly efficacious oral weight-loss agent, which acts centrally to suppress appetite and increase satiety; and (2) Early experimental data on topiramate suggested that it could significantly reduce colonic tissue damage in animal models of IBD	Large retrospective cohort study using United States administrative claims data (n = 1731): Compared new users of topiramate with users of other anticonvulsant/anti-migraine medications	Topiramate use was not associated with markers of IBD flares including steroid prescriptions (HR = 1.14, 95%CI: 0.74-1.73), initiation of biologic agents (HR = 0.93, 95%CI: 0.39-2.19), abdominal surgery (HR = 1.04, 95%CI: 0.17-6.41), or hospitalization (HR = 0.86, 95%CI: 0.62-1.19)	Crocket *et al*[83], 2014
Bariatric endoscopic applications	Intragastric balloon: Weight loss achieved through endoscopic bariatric interventions might achieve the same effect on outcomes in IBD as in other autoimmune diseases, but has not been studied	Case report of UC patient	UC worsened after insertion of an intragastric balloon for the treatment of obesity	Manguso *et al*[88], 2008

Bariatric surgery: BMI ≥ 40 kg/m^2 or 35-39.9 kg/m^2 with obesity-related comorbidities and previously failed to achieve adequate weight reduction with non-surgical interventions	Bariatric surgery: (1) Several studies have demonstrated that bariatric surgery is likely feasible, safe, and effective weight loss strategy, that may lead to improved outcomes of IBD patients; and (2) No RCTs or prospective studies were found that compared the different bariatric procedures in patients with IBD	Case-control study of 85 IBD patients, matched to non-IBD patients with BS (n = 85): (1) 20 UC, 64 CD, 1 unclassified IBD; (2) BMI 41.6 ± 5.9 kg/m^2; and (3) 3 RYGB/73 SG/12 LAGB	Bariatric surgery is a safe and effective procedure in obese IBD patients: (1) At a mean follow-up of 34 mo, mean weight was 88.6 ± 22.4 kg; (2) Complications: 8 (9%); and (3) No difference was observed between cases and controls for postoperative complications (P = 0.31), proportion of weight loss (P = 0.27), or postoperative deficiencies (P = 0.99)	Reenaers *et al*[93], 2022
		Case-control study of 25 IBD patients who underwent BS, matched to IBD patients who did not undergo BS (n = 47)	IBD patients with weight loss after BS had fewer IBD-related complications compared with matched controls: (1) Median decrease in body mass index after bariatric surgery was 12.2; and (2) Rescue corticosteroid usage and IBD-related surgeries were numerically less common in cases than controls (24% *vs* 52%, OR = 0.36, 95%CI: 0.08-1.23; 12% *vs* 28%, OR = 0.2, 95%CI: 0.004-1.79)	Braga Neto *et al*[95], 2020
		Retrospective review (n = 20): (1) 13 UC, 7 CD; (2) BMI 50.1 ± 9 kg/m^2; and (3) 9 SG/7 RYGB/3 AGB/ 1 AGB to RYGB	BS is safe and mitigate IBD: (1) Weight loss: 14.3 ± 5.7 kg/m^2 or 58.9% ± 21.1%; (2) Complications: Early 7 (5 Dr, 1 PE, 1 WI), late 5 (2 Pnt, 2 VH, 1 MU), mortality 1 (unrelated); and (3) IBD status after BS: Remit 9, exacerbate 2, no change 9	Aminian *et al*[91], 2016
		Prospective case-control study (n = 6/101): (1) 1 UC, 5 CD; (2) BMI 40.6 ± 3.74 kg/m^2; and (3) 1 Maclean gastroplasty/1 SG + end colostomy/2 SG/2 SG + ileocecal resection	BS is safe and effective and IBD Rx decreasing: (1) Weight loss: 11.45 ± 2.8 kg/m^2 or 28.14% ± 6.6%; (2) Complications: Late 1 (1 vomiting/dysphagia); and (3) IBD status after BS: Remit 5, exacerbate 1	Colombo *et al*[105], 2015
		Prospective study (n = 10): (1) 2 UC, 8 CD; (2) BMI 42.6 ± 5.6 kg/m^2; and (3) 9 LSG/1 LAGB	BS is effective and safe: (1) Weight loss: 71.4 ± 5.9 EWL%; (2) Complications: Early 1 (1 SLL) late 4 (4 VitD); and (3) IBD status after BS: Remit 2, exacerbate 3, no change 3, improved 1	Keidar *et al*[106], 2015
		Retrospective case-control (n = 4): (1) 4 CD; (2) BMI 45 ± 5.3 (40-51) kg/m^2; and (3) 4 LSG	SG is safe in CD: (1) Weight loss: 32.8 ± 4.3 kg/m^2 or 60.2% ± 13.7% EWL; (2) Complications: Early 1 (1 SLB); and (3) IBD status after BS: Remit 4	Ungar *et al*[107], 2013
		Retrospective inpatient study (n = 493/15319): (1) 245 UC, 248 CD; (2) BMI 40.6 ± 3.74 kg/m^2; and (3) 48% SG, 35% RYGB, 17% LAGB	Complications: 0.4% malnutrition, 0.2% thromboembolism, 12% strictures, 0.6% renal failure; prior-bariatric surgery was associated with decreased IRR for renal failure, undernutrition, and fistulae formation in morbidly obese IBD patients [(IRR = 0.1; 95%CI: 0.02-0.3; P < 0.001), (IRR = 0.2; 95%CI: 0.05-0.8; P = 0.03), and (IRR = 0.1; 95%CI: 0.2-08; P = 0.03), respectively]	Sharma *et al*[108], 2018

IBD: Inflammatory bowel disease; QoL: Quality of life; CD: Crohn's disease; UC: Ulcerative colitis; RR: Risk ratios; IBDQ: Inflammatory bowel disease questionnaire; IRR: Incidence rate ratio; BS: Bariatric surgery; OR: Odds ratio; Dr: Dehydration; EWL: Excess weight loss; LAGB: Laparoscopic adjustable gastric banding; MU: Marginal ulcer; PE: Pulmonary embolism; Pnt: Pancreatitis; Rx: Treatment; SG: Sleeve gastrectomy; SLB: Staple line bleeding; SLL: Staple line leak; VH: Ventral hernia; VitD: Vitamin deficiency; WI: Wound infection; CI: Confidence interval; RCT: Randomized control trial; RYGB: Roux-en-Y gastric bypass.

careful consideration of a balanced diet is essential. One study involving IBD patients showed improvements in body weight, waist circumference, and steatosis when a mediterranean diet was prescribed[72]. There is no available data to provide evidence on the impact of overall calorie intake or supervised dietary weight loss on outcomes in patients with IBD.

Moreover, exercise may have a positive effect on regulating IBD activity. Moderate-intensity exercise has been shown to have anti-inflammatory effects through a variety of mechanisms, such as reducing visceral fat, decreasing the secretion of inflammatory adipokines, and reducing stress-induced dysfunction of the intestinal barrier[73]. A prospective study suggested that high levels of physical exercise may be associated with a lower risk of active disease in IBD patients[74]. However, it should be noted that these studies have primarily relied on subjective measures of well-being and have not utilized specific objective indicators of disease activity.

Pharmacologic treatment

Currently, anti-obesity treatment is often recommended for patients with a BMI of 30 kg/m^2 or above, or a BMI of 27 kg/m^2 or higher in the presence of obesity-related comorbidities[75]. There are several prescription drugs (orlistat, liraglutide, phentermine-topiramate and naltrexone-bupropion) available for weight loss, but there are no randomized controlled trials of these anti-obesity drugs in patients with IBD. The ESPEN/UEG guideline recommends that anti-obesity drugs may be utilized in patients with IBD, provided they are indicated for such use, with the exception of orlistat. Orlistat should be avoided due to its mechanism of action and associated adverse effects. Notably, a case report demonstrated the use of liraglutide, a glucagon-like peptide 1 (GLP-1) receptor agonist, in a patient with active CD and type 2 diabetes resulted in improved glycemic control and quality of life scores following a switch from insulin therapy[76]. A recent cohort study of patients with both IBD and type 2 diabetes showed that treatment with GLP-1-based therapies was linked with a lower risk of adverse clinical events, defined as a composite of the need for oral corticosteroid treatment, TNF-α-inhibitor treatment, IBD-related hospitalization, or IBD-related major surgery, as compared to treatment with other antidiabetic therapies [adjusted incidence rate ratio (IRR) = 0.52, 95%CI: 0.42-0.65)[77]. These observations suggest that GLP-1 based therapies may be a novel treatment option for IBD. Naltrexone and bupropion individually may have anti-inflammatory properties. Two small uncontrolled studies of IBD patients showed that naltrexone alone reduced disease activity and induced endoscopic response[78]. The initiation of low-dose naltrexone in IBD patients has been found to result in a decrease in the prescription of several medications that are typically deemed crucial for the treatment of IBD[79]. Bupropion has been associated with clinical improvement in case reports and case series in IBD patients[80,81]. Phentermine-topiramate is a weight-loss drug that has demonstrated highly efficacy in promoting weight loss in obese individuals. While the precise mechanisms are still being investigated, preclinical studies have provided evidence supporting the anti-inflammatory properties of both topiramate and phentermine. Early experimental evidence indicated that topiramate could significantly reduce colonic tissue injury in animal models of IBD. However, these findings were not corroborated in a subsequent retrospective cohort study conducted in human subjects[82,83]. A phase 2 clinical trial is currently in progress to investigate the effectiveness and safety of phentermine-topiramate in obese biologic-treated patients with UC (NCT04721873). Sodium-glucose linked transporter 2 inhibitor is the new glucose lowering drug, which has shown beneficial effects on the obesity and heart failure as well as type 2 diabetes[84]. This drug significantly improved acetic acid-induced IBD in animal models by activating autophagy signaling, inhibiting apoptosis and pro-inflammatory cytokines, lowering oxidative stress, and increasing wound healing[85-87].

Bariatric endoscopic applications

Intragastric balloon therapies are a minimally invasive and temporary treatment for inducing weight loss in obese patients. While this approach has been used successfully in the management of obesity, there is limited research on the effectiveness and safety of intragastric balloon therapy in patients with IBD. Although a small series has examined the application of intragastric ballooning in patients with IBD, reports that detail the long-term outcomes associated with weight loss and complications are lacking. The use of intragastric balloon is currently contraindicated for IBD patients due to concerns about the potential for exacerbation of symptoms and complications. A case report demonstrated that the placement of an intragastric balloon for weight loss may exacerbate symptoms of UC[88]. Further, there is currently a scarcity of high-quality evidence regarding the efficacy of other endoscopic procedures for weight loss in patients with IBD.

Bariatric surgery

For the general population, in obese patients with BMI ≥ 40 kg/m^2 or 35-39.9 kg/m^2 with obesity-related comorbidities and previously failed to achieve adequate weight reduction with non-surgical interventions, bariatric surgery is superior to lifestyle and diet interventions and reduces mortality[89]. There are several different techniques for bariatric surgery, the most common being a Roux-en-Y gastric bypass (RYGB) and a sleeve gastrostomy (SG). These are now mainly performed laparoscopically.

Although data are sparse, bariatric surgery may be a viable, effective, and safe weight loss approach that could potentially result in improved outcomes for patients with IBD[90-93]. In most cases revised from two systematic reviews, weight loss resulting from bariatric surgery can be beneficial in achieving remission, reducing disease activity, and decreasing medication dependence in patients with IBD[92, 94]. Bariatric surgery resulted in similar weight loss and risk of complications in IBD patients as in non-IBD patients. A case-control study that included 88 bariatric procedures performed on 85 IBD patients, matched 1:2 for age, sex, BMI, hospital of surgery, and type of bariatric surgery with non-IBD patients who underwent bariatric surgery, found that bariatric surgery was a safe and effective intervention for weight loss, producing similar results to those observed in the control group over a 2-year period[93]. A case-control study involving 25 patients with IBD who received bariatric surgery, and matched with IBD patients who did not undergo bariatric surgery, found that the usage of rescue corticosteroids and the requirement for IBD-related surgery were less prevalent in the former group than in the controls [95]. However, long-term effects of bariatric surgery in IBD patients are poorly understood.

Although conclusive findings have yet to be derived from randomized trials, current evidence suggests that SG may confer an advantage over RYGB in patients with IBD, as SG solely involves the stomach and thus may minimize the risk of small intestinal bacterial overgrowth[90,96]. By avoiding anatomical alterations in the small intestine, the risk of complications such as strictures, abscesses, and fistulas may be reduced and future IBD-related surgeries may be simplified.

De-novo IBD after bariatric surgery

The development of *de-novo* IBD subsequent to bariatric surgery has been reported in several studies. The potential mechanisms underlying this association include exposure to viable toxins as a result of anatomical alterations, increased release of cytokines due to changes in adipose tissue, and alterations in the gut microbiome[95,97]. A Danish nationwide population-based cohort study has reported that bariatric surgery is related to an increased risk of developing new-onset CD, but not UC[98]. After bariatric surgery, the onset of IBD symptoms varied from 1 mo to 16 years[97]. In a case series, it was found that 44 patients who had previously received bariatric surgery, with RYGB being the most common procedure, developed *de-novo* IBD after a median latency period of 7 years[99]. Recent metal-analysis including 149385 patients reported that the pooled odds ratio for the *de-novo* IBD following bariatric surgery is 1.17[100]. Thus, the possibility of *de-novo* IBD should be taken into account as a potential cause of symptoms such as abdominal pain and diarrhea in patients who have undergone bariatric surgery.

CONCLUSION

Both obesity and IBD are rapidly increasing in modern society, and the proportion of obesity among IBD patients is also reported to be higher now than that in the past. There are claims that obesity contributes to the pathogenesis of IBD or that there are common factors contributing to both diseases, such as dysbiosis, but it is still insufficient to know the causal relationship or direction between them. Although data to assess the effect of obesity on outcomes in IBD are sparse and inconclusive, obesity may have a protective effect in CD and a poorer prognosis in UC. Obesity can clearly affect the treatment of IBD or surgery. Therefore, clinicians need to be aware that obesity can affect the treatment response to drugs and increase surgical complications. Although there are few studies, obesity treatment appears to have the potential to have a relatively favorable effect on IBD outcomes. Bariatric surgery appears to be relatively safe and effective in obese IBD patients. Therefore, it is necessary to compare the benefits and side effects of bariatric surgery for IBD patients. Some patients develop new onset IBD, especially CD, after bariatric surgery, so clinicians are advised to keep this in mind.

FOOTNOTES

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REVIEW

Role of autoantibodies in the clinical management of primary biliary cholangitis

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Abstract

Primary biliary cholangitis (PBC) is a chronic cholestatic liver disease characterized by immune-driven destruction of small intrahepatic bile ducts leading a proportion of patients to hepatic failure over the years. Diagnosis at early stages in concert with ursodeoxycholic acid treatment has been linked with prevention of disease progression in the majority of cases. Diagnosis of PBC in a patient with cholestasis relies on the detection of disease-specific autoantibodies, including anti-mitochondrial antibodies, and disease-specific anti-nuclear antibodies targeting sp100 and gp210. These autoantibodies assist the diagnosis of the disease, and are amongst few autoantibodies the presence of which is included in the diagnostic criteria of the disease. They have also become important tools evaluating disease prognosis. Herein, we summarize existing data on detection of PBC-related autoantibodies and their clinical significance. Moreover, we provide insight on novel autoantibodies and their possible prognostic role in PBC patients.

Key Words: Primary biliary cholangitis; Anti-mitochondrial antibodies; Primary biliary cholangitis-specific antinuclear antibodies; Anti-sp100; Anti-gp210; Prognosis

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Core Tip: The diagnosis of primary biliary cholangitis (PBC) relies on the detection of disease-specific autoantibodies, including anti-mitochondrial antibodies and disease-specific antinuclear antibodies targeting sp100 and gp210. In this review, we summarize existing data on detection of PBC-related autoantibodies and their clinical significance. Moreover, we provide insight on novel autoantibodies and their possible prognostic role in PBC patients.

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INTRODUCTION

Primary biliary cholangitis (PBC), known until 2014 as primary biliary cirrhosis, is a chronic autoimmune cholestatic liver disease, its main features being presence of anti-mitochondrial antibodies (AMA), female predominance and progressive destruction of small intrahepatic bile ducts[1]. A proportion of PBC patients progresses over the years to fibrosis and eventually to cirrhosis leading to hepatic failure. Diagnosis at early stages in conjunction with ursodeoxycholic acid (UDCA) treatment has been linked with prevention of disease progression in the majority of cases. Over the years, significant progress has been achieved in the armamentarium used for disease diagnosis and prognostication, including autoantibody testing and on-treatment prognostic indicators[1].

Originally PBC was reported in 1851 in a woman with jaundice and xanthomata and its clinical phenotype was described in 1949[2,3]. The first association of PBC with autoantibodies was reported by Mackay[4] in 1958 in a case with high titers of circulating complement-fixing antibodies to liver, kidney and other human tissue antigens[4]. Later on, in 1965, Walker *et al*[5] have reported the presence of AMA by indirect immunofluorescence (IIFL) in patients with PBC[5].

The discovery of AMA in conjunction with advances in their diagnostic testing and increased disease awareness has led to diagnosis at earlier stages. Moreover, treatment with UDCA, which is the standard of care for naïve PBC patients has been associated with improved long-term survival. All these factors have significantly contributed to the increase in prevalence rates throughout the years[6,7]. The highest incidence and prevalence rates have been reported in Europe and the United States (incidence and prevalence rates range from 3.3 to 32 per million person-years and 19 to 402 per million respectively)[8-11]. Discrepancies between regions may reflect differences in patients' accessibility to healthcare services, increased disease awareness depending on physician's expertise and widespread use of AMA and antinuclear antibody (ANA) testing during diagnostic work-up. Still, it is debatable whether increases in prevalence and incidence rates represent true changes over the years.

PBC is considered a prototype autoimmune disease, based on the abundance of AMA, female predominance and increased rate of other autoimmune diseases. Genetic and environmental factors are regarded as key players in the induction of immune tolerance loss to biliary epithelial autoantigens, a notion well appreciated also on work performed in animal models of the disease[12-15].

Higher disease rates between family members, especially siblings, formed the initial pathogenetic link between genetics and PBC. Genome wide association studies from Europe, North America, Japan and China have identified human leukocyte antigens (HLA) and non-HLA alleles that confer susceptibility to PBC, though discrepant results between ethnicities are apparent[16]. The majority of reported loci are encoding proteins implicated in the control of immune response mechanisms, including HLA, interleukin (IL)-12 production, B and T cell activation, interferon (IFN)-γ production and production of immunoglobulins. The contribution of genetics is also emphasized by the fact that monozygotic twins display significantly higher concordance rates compared to dizygotic twins (63% *vs* 0%)[17]. Still, lack of remarkable concordance rates between identical twins in PBC strongly supports the implication of environmental factors and epigenetics.

Numerous studies have revealed the important contribution of environmental exposures, including chemical xenobiotics, pollutants, cosmetics and also infectious agents, in immune tolerance loss and in the initiation of disease process[18-21].

Studies on animal models of the disease have underlined the likely input of specific environmental factors in the initiation and perpetuation of disease progression, adding further support for their significant input in genetically prone individuals[15].

DIAGNOSIS OF PBC

The cornerstone for PBC diagnosis is the detection of AMA and/or PBC-specific ANA when investigating a patient with cholestasis (Table 1)[6]. It is important to stress that AMA are one of the few autoantibodies being included in diagnostic criteria of a disease[6]. Liver histology is essential when AMA are not detected in a patient with cholestasis with high suspicion of PBC, or when indications of variant forms exist[6,22].

AUTOANTIBODIES

AMA

Since their first description in 1965 by Walker *et al*[5], AMA have been uninterruptedly used as the most reliable marker for the diagnosis of PBC[5]. This landmark paper did not showcase only the close association of AMA with PBC, but provided also a straightforward technique for its detection, which has been routinely used up to this time, namely IIFL[5]. Later on, in 1968, a trypsin-sensitive antigen was identified by Berg *et al*[23] to react *in vitro* with sera from PBC patients, which was located in the inner mitochondrial membrane and was called M2[23]. The same group proposed a classification of AMA antigens from M1 to M9[24]. This nomenclature was subsequently abandoned, as several studies have failed to link PBC with any of these antigens, except for M2[25,26].

M2 antigens consist of components of the 2-oxo-acid dehydrogenase multienzyme complexes; namely, pyruvate dehydrogenase (PDC), 2-oxoglutarate (OGDC) and branched-chain 2-oxo acid (BCOADC)[27]. Each complex is formed by several copies of at least three enzymes (E1, E2 and E3) that form high molecular-weight multimers and are localized in the inner mitochondrial membrane. The E2 enzyme, based at the structural core of the complex, contains the lipoyl group, which is bound to lysine and plays significant role in the catalytic cycle[27].

The next major breakthrough was the cloning and sequencing of a 74 kDa mitochondrial autoantigen, that led to the identification of E2 subunit of PDC-E2 as the major autoantigen in PBC[27,28]. Several reports were able to establish the fine characteristics of response to antimitochondrial antigens in PBC patients[29]. In detail, up to 95% of PBC patients show reactivity to PDC-E2 and to lesser extend (50%-70%) towards OGDC-E2 and BCOADC-32[29]. Few AMA positive PBC patients react only towards PDC-E2 and even less solely to OGDC-E2 or BCOADC-E2. The predominant anti-M2 reactivity is the one against the E2 component of PDC and also BCOADC and OGDC enzyme complexes, while less commonly reactivity to PDC-E1a and PDC-E1b subunits and also the dihydrolipoamide dehydrogenase (E3)-binding protein has been reported[29]. It is of interest that the E2 subunit of the OADC is highly conserved between species and also between various complexes. All immunodominant epitopes include a ExDKA motif with a lipoic acid attached to K at position 173, which is essential for T-cell antigen recognition[30].

Several studies have tried to elucidate mechanisms involved in loss of tolerance towards 2-OADC components located on the inner mitochondrial surface of biliary epithelial cells (BECs), finally leading to BECs injury and are reviewed elsewhere[31]. A multi-lineage loss of tolerance against major AMA epitopes appears to be one of the key features leading to progressive destruction of bile ducts in PBC where both CD4+ and CD8+ T cells orchestrate an immune response against the PDC-E2 complex in the liver as well as in the periphery[31]. IL-12 and IFN-γ prevail in PBC inducing Th1 immune responses at initial stages of the disease[32]. Perpetuation of liver injury during the disease process is linked with skewing towards a Th17 phenotype[31,32]. T follicular helper cells, known to assist B cell-specific antibody production, were found in increased numbers in livers of PBC patients, while T regulatory cells, that promote self-tolerance, were found numerically or functionally impaired in PBC patients[31].

Over the years, the role of BECs has been upgraded from regulators of fluidity and alkalinity of bile, to active participants of innate and adaptive immune responses contributing to bile duct injury and evolution of liver disease[1,33]. Why BECs are the targets of autoimmune attack, considering that PDC-E2 is not confined solely to these cells but is abundant in mitochondria of all nucleated cells, remains elusive. Several studies have proposed a hypothesis according to which small BECs, by undergoing apoptosis, transfer immunologically intact PDC-E2 to apoptotic bodies and form an apotope (antigenic epitope). This apotope in concert with macrophages and AMA can induce locally acting proinflammatory cytokines resulting in inflammation and surrounding apoptosis in PBC[14].

Methods for AMA detection

IIFL: Since its discovery, IIFL is considered the "gold" standard technique for the detection of AMA and titers above 1/40 are regarded as positive[6,22]. The preferred tissue for the detection of AMA is liver, kidney and stomach substrates, which displays a fluorescence pattern unique to AMA. AMA stains the distal tubules on kidney sections, which contain more mitochondria compared to the proximal tubules, while it produces a bright granular pattern on gastric sections and a faint cytoplasmic pattern on liver sections[34]. Alternatively, on a substrate of human larynx epithelioma cancer cell line (HEp-2) cells, AMA give a diffuse, granular cytoplasmic pattern, that is not consistent with other methods of AMA

Table 1 Autoantibodies detected in primary biliary cholangitis and their clinical significance

Autoantibodies	Method	Prevalence	Specificity	Diagnostic relevance	Clinical significance
AMA	IIFL, ELISA, Blot	90%-95%. Varies according to method used	High (90%)	Yes	AMA presence and titer not associated with severity of PBC or prognosis of PBC
Antinuclear antibodies				Yes	
Against nuclear envelope antigens: Anti-sp100; anti-PML; anti-sp140; anti-SUMO	IIFL (MND pattern), ELISA	30%-50%	Very high (approximately 98%)	Useful in AMA-negative patients	Anti-sp100 is associated with more severe disease, faster disease progression and worse outcome
Against nuclear pore complex antigens: Anti-gp210; anti-np62; anti-LBR	IIFL (Rim-like perinuclear pattern; RL/M), ELISA, Immunoblot	30%-50%	Very high. Up to 100%	Useful in AMA-negative patients	Anti-gp210 is associated with more severe disease and worse outcome
Anticentromere antibodies	IIFL	10%-30%	Low	No	Limited data on their role as prognostic factor for complications related to portal hypertension
Anti-KLHL12 antibodies Anti-HK-1 antibodies	ELISA Immunoblot	22%-36%	Very high	Yes. Useful in AMA-negative pts	Anti-HK1 is associated with worse outcome (needs validation)

sp100: Nuclear body speckled 100 kDa; sp140: Nuclear body speckled 140 kDa; SUMOs: small ubiquitin-related modifiers; PML: Promyelocytic leukemia proteins; LBR: Lamin B receptor antibody; KLHL12: Anti-kelch-like 12 protein; HK-1: Anti-hexokinase 1; AMA: Anti-mitochondrial antibodies; MND: Multi nuclear dot pattern; ELISA: Enzyme-linked immunosorbent assay; IIFL: Indirect immunofluorescence; PBC: Primary biliary cholangitis.

detection (*i.e.* IIFL with triple substrate or molecular based assays) and its use as a single method for AMA detection is not recommended. Still, HEp-2 cells is recommended to be used in parallel with the triple rodent substrate for the detection of PBC-specific ANA[34].

Data from few old studies have shown that AMA are not restricted to a specific immunoglobulin (Ig) G subclass, though IgG3 appears to be the most predominant[35,36].

Solid phase assays: The identification of AMA molecular targets has revolutionized the diagnostic approach of patients with PBC, since new molecular-based assays have been developed, which relay on the use of recombinant or purified antigens. Amongst them are microtiter plate Enzyme-linked immunosorbent assay (ELISA), chemiluminescence and fluorescent bead-based assays[37-43]. Commercially produced ELISA's have substantially gained ground over the years, considering they offer high grade of standardization and automation and don't require skilled personnel either for their application or interpretation[44].

Gershwin's group have created a recombinant fusion protein (MIT3), consisting of the major immunodominant epitopes of the three main AMA targets (PDC-E2, BCOADCE2, OGDC-E2)[41]. An ELISA utilizing MIT3 had increased sensitivity compared to IIFL and ELISAs with conventional anti-M2, as it allowed the identification of AMA in 30%-50% of previously AMA-negative samples[39,45]. An improved technique, based on the coupling of the three recombinant mitochondrial autoantigens (PDC-E2, BCOADC-E2 and OGDC-E2) with beads, found 20% of AMA-negative by IIFL patients to be positive, while 100% of these new AMA positive patients were revealed as ANA positive[46]. The development of another assay (anti-M2-3E ELISA),which included antibodies to MIT3 and purified PDC to allow detection of additional less immunodominant mitochondrial antigens, like PDC-E1a and E1b, increased further the diagnostic accuracy of AMA compared to IIFL, conventional anti-PDC ELISA and also the anti-MIT3 ELISA[37].

Several studies have suggested that investigating individual AMA isotypes (IgG, IgM, IgA) could be of clinical relevance, as different isotypes may correlate with biochemical, clinical and histological features of the disease. As a secretory immunoglobulin, IgA is abundant in mucous membranes, while IgA AMA have been detected in bile, saliva and urine samples of PBC patients and have been suggested to contribute to bile duct injury[47,48]. Of relevance, PDC-E2 specific dimeric IgA, but not IgG resulted in induction of caspase activation in Madine-Darby canine kidney cells transfected with the human polymeric Ig receptor, in an experimental study[49]. Studies assessing the possible clinical significance of IgA AMA in PBC patients have produced conflicting results up to the present[39,50-52]. Even if we cannot recommend IgA AMA testing based on these remarks, we should stress that a minority of PBC patients (2%-3%) have only AMA of IgA isotype[39]. In this context, if a patient is suspected to have PBC based on clinical and biochemical features and IgG AMA are not detected, he should be tested for AMA of IgA isotype.

A study from China, has suggested that salivary AMA-M2, tested by ELISA is a useful biomarker for the diagnosis of PBC. It is a non-invasive method, providing high specificity, as salivary AMA-M2 were detected only in serum AMA-M2 positive PBC patients and none of the controls[53].

In PBC, SDS-PAGE followed by western blotting has been used in the past as a sensitive and specific method in identifying individual mitochondrial antigens based on their molecular weight (74 kDa band for PDC-E2, 52 kDa band for BCOADC-E2 and 48 kDa band for OGD-E2). Preparations of mitochondrial fractions of primate and/or liver or bovine heart are usually used as source of AMA antigens, though recombinant proteins are suggested to produce less background[54,55]. Immunoblotting with recombinant protein results in 91% positivity in sera from PBC patients, compared to 81% when recombinant PDC-E2 fusion protein is utilized[54]. Similar to ELISAs, the fact that a small portion of PBC patients demonstrate reactivity only to BCOADC-E2 and/or OGDC-E2 could account for this variance in sensitivity. A study applying computer-assisted imaging technology showed correlation between AMA titers by IIFL and number and intensity of immunofixed 1-OADC bands in sera from PBC patients[55]. However, immunoblotting is time consuming and in routine practice its usage has been abandoned giving space for the most reliable and fully automated antigen-specific assays or automated IIFL.

Data from a recent study suggests that M2-AMA dot blot is more specific than IIF-AMA[56]. This study demonstrated increased sensitivity and specificity with increasing number of M2-AMA specificities detected.

In recent years automated particle-based multi-analyte technology (PMAT) assays have been used for the detection of autoantibodies, including those related to PBC. Data evaluating the performance of this assay have been convincing and commercial assays based on this technology are widely available, as they allow the concurrent detection of several antigen-specific autoantibodies[57].

AMA AND THEIR CLINICAL CORRELATIONS

AMA is the most characteristic feature of PBC, as up to 95% of patients are tested positive for these autoantibodies. A 2014 meta-analysis including 24 studies showed that the pooled sensitivity and specificity of AMA in the diagnosis of PBC is 84.5% and 97.8%, respectively[58]. The specificity of AMA for PBC has been initially revealed in two small longitudinal studies, where the majority of AMA positive patients with no evidence of cholestatic liver disease developed full blown PBC[59,60]. Still, recent studies have shown a small proportion of AMA positive individuals to develop PBC through the years[61,62]. These data should be interpreted with caution, as follow up duration might have been not long enough in these latter studies to allow clinical presentation of PBC that is known to progress slowly.

Data from Sun *et al*[63], though, have shown that 80% of AMA positive individuals with normal alkaline phosphatase have histological features of PBC, stressing that cholestasis is not a prerequisite for the establishment of PBC[63]. The pathogenic role of AMA is further supported by the case of two newborns where liver disease had developed after transfer of AMA from their mothers *via* the placenta, while liver pathology subsided when the autoantibodies disappeared.

However, AMA have been reported in up to 1% of healthy individuals[64,65]. Considering the substantially lower PBC prevalence, only a small percentage of those AMA positive individuals is going to advance to PBC. Moreover, longitudinal data on AMA kinetics in patients transplanted for PBC show these autoantibodies to persist, though biochemical or histological features of PBC recurrence have been reported in 36% 10 years post liver transplantation[66,67]. As autoantibodies can arise years before disease presentation only long-term observational studies spanning a period of decades will be able to elucidate this issue further.

Kisand *et al*[68] have suggested that development of PBC might arise in those who have or will develop high titer AMA overtime of various specificities and subclasses compared to AMA positive individuals with low titer antibodies of only one Ig class reactivity[68].

Whether AMA titers is a prognostic indicator for PBC needs to be assessed further, as several studies up to the present have produced conflicting data. Early studies from the 80s and 90s have shown AMA titers to correlate to disease activity and progression[69,70]. Another study demonstrated that PBC patients had significantly higher AMA titers, tested by IIFL and higher anti-PDC-E2 avidity compared to AMA positive individuals with normal biochemistry[71]. Moreover, Gabeta *et al*[39] have demonstrated IgG and IgA AMA titers to positively correlate with the Mayo risk score[39].

Whether testing by IIFL for individual IgG AMA subclasses could assist in identifying prognostic features of PBC patients remains obscure. Another study stressed that AMA of the IgG3 subclass positively correlate with more advanced liver disease, as manifested by higher frequency of cirrhosis and higher Mayo risk score[36].

Furthermore, treatment with UDCA was associated with a decrease in AMA titers in one of the first trials of UDCA in PBC patients, while in a recent study from China, response to UDCA treatment at 1 year was linked to decreased AMA titers[72,73]. However, several other studies failed to prove AMA titers or their longitudinal changes as prognostic markers of PBC progression[74-76]. In line with this, a

small study on 9 asymptomatic PBC patients with inadequate response to UDCA, who continued with combination of UDCA and fenofibrate, showed a decrease in AMA titers in 4 of these patients. These data could suggest that AMA production may be regulated by peroxisome proliferator-activated receptor α in PBC patients. Still, this is a hypothesis that needs to be confirmed in the future, as it was not reproduced by other studies[77].

Anti-M2 AMA can also occur in the context of overlapping diseases, such as the autoimmune hepatitis/PBC variant, metabolic associated fatty liver disease/PBC variant, viral hepatitis (hepatitis B virus, hepatitis C virus)/PBC variant and other rheumatic diseases, such as systemic sclerosis[6,78,79]. Their presence should be evaluated in the appropriated clinical context.

ANA IN PBC

Various ANA specificities have been reported in up to 70% of PBC patients[80]. HEp-2 cells are preferred compared to the triple tissue substrate for the detection of ANA, as large nuclei and high rate of mitosis of these cells permit the discrimination of different staining patterns[34]. A variety of staining patterns, often coinciding, have been described in PBC patients, including rim-like (RLM), nuclear dot, speckled, homogeneous and centromere staining pattern[81,82]. For diagnostic purposes, ANAs are categorized into those not specific for PBC and those specific for the disease.

According to the official categorization of ANA patterns issued by the International Consensus on ANA Patterns initiative, 2 IIFL patterns are defined as PBC-specific, namely the multiple nuclear dot (MND) pattern (AC-6) and the punctuate nuclear envelope pattern (AC-12)[83].

ANA not specific for PBC

Anticentromere antibodies (ACA) and those against extractable nuclear antigens (anti-ENA), recognizing various molecular targets, including nuclear ribonucleoproteins (nRNP), ribosomal phosphoproteins and cellular enzymes such as DNA topoisomerase I (Scl-70) and histidyl-tRNA synthetase (Jo-1) are non-specific ANA and can be detected up to 30% of PBC patients, at times indicating co-existing autoimmune rheumatic diseases[80,84].

Originally, IIFL on HEp-2 cells was used for ACA detection and counter immunoelectrophoresis on thymic and spleen extracts for anti-ENA detection. More sensitive methods, like ELISA and immunoblot were developed for the detection of various molecular targets (*i.e.* nRNP, Sm, SSB/La, SSA/Ro 60 and 52). Their application has revealed anti-ENA reactivities in up to 30% of PBC patients, anti-SSA/Ro-52kD being the most frequent one. The presence of anti-SSA/Ro-52 kD was associated with active and advanced disease in one study[84]. ACA have been reported in 20%-30% of PBC patients and 80% of patients with a PBC/Systemic Sclerosis overlap syndrome[81,84-86].

The diagnostic and clinical significance of ACA positivity in patients with PBC without SSc has recently been under investigation, though with discrepant results[86-88]. Data from two studies have suggested ACA to be a predictive factor for the development of complications related to portal hypertension in PBC patients, though not for the progression to liver failure[86,88]. As both studies were conducted in PBC patients of Asian origin, large scale data are needed to explore this hypothesis further.

PBC-specific ANA

PBC-specific ANA have been reported in up to 50% of PBC patients, with wide variation in the prevalence rate between studies depending on the method used[80]. They demonstrate high specificity, though low sensitivity for PBC and is a valuable tool during diagnostic work-up of patients with suspicion of PBC, especially in those tested negative for AMA[6,22]. Their identification is optimized when HEp-2 cells are used as a substrate, as these cells have large nuclei and during their mitotic phase ACA stain their chromosomes[34].

PBC-specific ANA display two distinct immunofluorescence patterns: A perinuclear/RLM and a MND pattern[80,83]. The RLM pattern gives a distinctive punctuated pattern of the nuclear surface that corresponds to a nuclear complex. This pattern is generated by reactivity to nuclear pore complexes (NPC), which are multi-protein structures that mediate nucleocytosplasmic transport. Gp210 is the main antigenic targets in PBC and is currently used for diagnostic purposes. Less immunodominant antigens, not incorporated in the diagnostic work-up, are those of nucleoporin p62 and lamin B receptor. The MND pattern consists of 3-20 dots scattered throughout the nucleus, but sparring the nucleoli and is produced by reactivity to the nuclear body speckled 100 kDa (sp100) protein and the promyelocytic leukemia (PML) protein, while more recently sp140 and small ubiquitin-related modifier (SUMO) protein has been reported as additional antigenic targets[80,89,90].

The visualization of ANA can be hindered by AMAs presence and other ANA specificities[34]. This could justify the perception that prevailed based on the results of early studies reporting of the presence of PBC-specific ANA especially in AMA-negative patients[81]. For example, the MND pattern can be easily mistaken with that produced by ACA; the anti-MND pattern consist of dots of various size and numbers, while the ACA pattern is characterized by dots of equal size. ACA stain the cells'

chromosome, whereas anti-MND don't.

A multicenter study showed that the diagnostic performance of the conventional IIFL improved further by the use of individual IgG (IgG1-4) isotypes instead of anti-total IgG sera, leading to increased recognition of PBC-specific ANAs[91]. In detail, 65% and 15% of PBC patients were MND and/or RLM positive using IgG isotype-specific antisera and anti-total IgG antiserum respectively[91].

The identification of the molecular targets of PBC-specific ANA has led to the development of molecular based techniques, including ELISA and immunoblot, that are more sensitive and observer-independent[80,92,93]. Accordingly, Invernizzi *et al*[92] demonstrated reactivity to NPC by immunoblotting in 22% of 105 patients known to be ANA negative by IIFL. There was no significant difference in the percentage of anti-NPC positive amongst those being AMA positive and negative PBC patients with the use of immunoblotting[92]. Similarly, Muratori *et al*[81] have reported on the increased sensitivity of ELISA in detecting PBC-specific ANAs compared to IIFL, though they were more often detected in AMA negative compared to AMA positive PBC patients[81].

Still, a major limitation of the molecular based assays is the selection of specific targets and exclusion of others that might be of potentially equal importance, especially if reactivity to those subdominant antigens is of diagnostic relevance (such is the case of single specificities).

The sensitivity of anti-gp210 has been reported between 5.71% and 55.88%, whereas specificity ranged from 61.70% to 100% depending on the method used and the population studied[94-98].

ANA AND THEIR CLINICAL CORRELATIONS

Several studies have evaluated whether PBC-specific ANAs have a prognostic role in PBC patients. Discrepant results between studies can be justified by differences in study design (*i.e.* methods to evaluate the presence of antibodies, different antigenic preparations used), as well as heterogeneity of patients investigated[86,94].

Early studies, based mainly on IIFL detection of autoantibodies, had failed to establish a link between presence of PBC-specific ANAs and disease' prognosis[99,100]. During the last 20 years, several reports relaying mostly on ELISA, western blot and line blot data, delineated the correlation of PBC-specific ANAs with more severe disease and worse outcome, though differences between studies exist. Most studies underline that positivity for anti-NPC antibodies and especially for anti-gp210 are associated with more severe disease and worst disease outcome[81,87,91,92,97,101].

One of these studies using IIFL for the identification of autoantibodies, revealed anti-RLM of the IgG3 isotype to correlate with more severe liver disease[91]. A meta-analysis encompassing 5 studies in Asian patients demonstrated anti-gp210 antibodies to be associated with worst disease outcome and suggested their use as optimal predictors of PBC prognosis at the time of diagnosis[102].

Nakamura *et al*[103] reported that persistently positive anti-gp210 antibodies during follow up in Japanese PBC patients under UDCA treatment confer a strong risk for progression to end-stage liver disease, whereas these patients had histologically more severe interface hepatitis, lobular inflammation, and ductular reaction[103]. These findings were not confirmed in a study assessing autoantibody patterns during follow up in Greek PBC patients[104].

Anti-np62 antibodies are detected in 22%-31% of PBC patients, though their significance is not well documented and their diagnostic relevance remains unclear[105,106]. A Japanese study suggested that anti-np62 antibodies are associated with advanced disease. These results need to be confirmed by other studies[105].

Anti-sp100, the most frequent amongst antibodies displaying the MND pattern, is detected in 8%-44% of PBC patients, its specificity ranging from 63.8% to 100%[81,107-109]. The pooled sensitivity of anti-sp100 for PBC has been estimated at 23.1% and the pooled specificity at 97.7%[98]. Importantly, subgroup analysis could not identify significant differences in pooled specificity across the strata of geographical regions or across various methods used for their identification[98].

The prevalence of anti-lamin B receptor antibodies have been reported in 9%-15% of PBC patients and are considered highly specific[105,110]. Given their rarity and their unknown prognostic role, they are not routinely tested in every day clinical practice.

Anti-MND and anti-sp100 antibodies tested by IIF and by ELISA respectively have been associated with more severe disease, as attested by biochemical and histological features, faster disease progression and worse outcome[91,107]. Moreover, anti-MND of the IgG3 isotype was associated with longer disease duration and more severe histological picture[91].

Autoantibodies against PML are less frequently detected in PBC patients (12%-19%), and largely co-exist with anti-sp100, though cases with single reactivity to PML have been also reported[93,107]. Double reactivity to sp100 and PML was suggested to be associated with unfavorable outcome of PBC patients[93,107]. SUMOs have been exclusively linked to presence of anti-sp100 and anti-PML, suggesting antigen spreading as a possible mechanism for anti-SUMO generation[90].

Züchner *et al*[107] reported anti-sp100 levels to remain stable during the course of the disease. Of interest, alteration in the sp100 epitope recognition pattern in some patients during the natural course of the disease was noted in a proportion of patients under UDCA treatment, which indicates that UDCA

can modify immunoglobulin expression[107]. In accordance with these findings are those from a study in 110 Greek patients, where serial determination of autoantibodies titers demonstrated that a decrease in anti-sp100 levels was associated with response to UDCA treatment and improvement of the Mayo risk score[104]. Akin to AMA, PBC-specific ANA are shown to persist after liver transplantation without signs of disease recurrence[106,111].

Few studies have suggested infectious agents to have acted as potential triggers for the development of anti-MND antibodies in PBC patients. Bogdanos *et al*[112] have demonstrated a strong correlation of anti-sp100 reactivity and presence of AMA only in those PBC patients with recurrent urinary tract infections[112]. Molecular mimicry between T-cell epitopes, first implicating mitochondrial and afterward nuclear proteins, has been proposed[113].

A study conducted in a Han Chinese population demonstrated significant genetic predisposition for sp100 but not for gp210. In detail, HLADRB1*03:01, DRB1*15:01, DRB1*01, and DPB1*03:01 alleles were associated with antibody production against sp100[114]. Whether this difference is indicative of diverse roles of anti-sp100 and anti-gp210 in PBCs pathogenesis needs to be elucidated in the future. Besides, as data of this study is confined to Chinese patients, these results need to be confirmed in other PBC cohorts.

AMA NEGATIVE PBC

The percentage of AMA negativity by IFL widely varies amongst studies, ranging from 5%-20%. Soon after the identification of the molecular targets of AMA and the development of molecular based assays, it has become apparent that such assays can considerably increase the sensitivity for AMA detection, leading to a significant drop of "true" AMA-negative cases[37,39,40,45,46,115]. By immunoblotting testing, IgG antibodies against PDC-E2 are detectable in 97% of AMA positive PBC and in 66% of AMA negative PBC[116].

Others have reported even higher prevalence of antigen-specific AMA in IFL AMA-negative PBC patients, reducing even more the percentage of AMA negative PBC[117-119]. In our hands, only 16% of IFL AMA-negative PBC patients had reactivity against mitochondrial autoantigens using a sensitive immunoblot technique[55].

Nevertheless, even when the most sensitive AMA tests are used, still a proportion of PBC patients may not have AMA. While some studies indicated that seronegative cases may convert to AMA seropositive over the years, some others have elegantly shown that there are still patients that will never develop these autoantibodies over the course of the disease. This is of fundamental importance as it implies that breaking of immunological tolerance to the mitochondrial autoantigens of AMA is not the sole cornerstone of PBC pathogenesis, and other non-mitochondrial antigen-driven mechanisms are potentially involved. It is also of diagnostic importance because it highlights the importance for the proper identification and routine use of diagnostic surrogate markers which can assist the diagnosis of PBC in AMA negative cases. It also raises the question as to whether AMA positive and AMA negative PBC are two entities with distinct characteristics or not.

It must be noted, that over the years and with the wealth of data provided, a consensus has been reached that "true" AMA-negative PBC is an entity indistinguishable from AMA-positive PBC, in terms of demographic, biochemical, clinical and histopathological features[117,118,120]. Though the total number of AMA negative cases with PBC included in various studies can be considered small, and despite the lack of multi-centre studies investigating in greater detail the features of AMA negative PBC, the current evidence supports the notion that the natural history as well as the prognosis of AMA-negative PBC is analogous to AMA-positive PBC[121,122]. Current guidelines indicate the therapeutical and clinical management of AMA negative PBC should be the same with AMA positive PBC. Early studies have reported that aside AMA status, some immunoserologic features may be different between AMA-negative and AMA-positive PBC, such as lower IgM and higher gamma globulin in the former rather than the latter group[117,118,120,123].

Several studies have published data reporting a significantly higher rate of positivity for ANA in AMA-negative compared to AMA-positive PBC. Original studies were based on IFL testing, while most recently such assays included ANA-specific antigen testing against sp100 and gp210. The wide range of ANA positivity is attributed to technical reasons, such as the sensitivity of the assay used for autoantibody detection and cohort selection bias or geographic/ethnic disparities. Early IFL studies reported the prevalence of ANA to range between 71% to 100%, in AMA-negative PBC patients, compared with 18% to 33% in AMA seropositive PBC patients[117,118,120,123].

The first point that at times is overlooked by the clinicians is that PBC-specific ANAs are not confined to AMA-negative PBC and therefore disease-specific ANA is by no means a characteristic feature of this form of the disease. It has now become apparent that this striking over-representation was due to the practical limitations imposed by IFL on tissue sections because ANA positivity could be obscured by the simultaneous presence of AMAs, which could perplex the reading by the immunodiagnostician. This is indeed true in week AMA positive cases were ANAs become more visible and can be present in frequencies comparable to AMA negative PBC cohorts[81]. With the advent of assays using as antigenic

substrate recombinant sp100 and gp210, a more accurate estimation of the respective autoreactivities has been achieved, though great variation is still seen amongst studies. In a consecutive cohort, Muratori *et al*[81] found ANA of any pattern in 53% of patients, including 27% with anti-Sp100, and 16% with anti-gp210 antibody reactivity[81].

Most studies fail to report significant differences of anti-gp210 and anti-sp100 between AMA-positive and AMA-negative PBC cohorts[124].

The relatively recent identification of novel autoantigens in AMA negative patients with PBC has provided further hints regarding the diagnostic and clinical challenges of AMA-negative patients with PBC and their relevance to the presence of disease-specific autoantibodies.

NOVEL AUTOANTIBODIES

During the last decade, antibodies against kelch-like protein (KLHL12) and hexokinase 1 (HK-1) were proposed as novel biomarkers in PBC patients, especially those lacking AMA[125,126]. While KLHL12 is a nuclear protein that is implicated in collagen export and ubiquitination of various proteins, HK-1 is an enzyme that is localized in the outer mitochondria membrane, phosphorylates glucose and modulates susceptibility to cellular apoptosis. Both display high specificity (≥ 95%), while their combination improved their overall sensitivity from 48.3% to 68.5% by ELISA and from 55% to 75% by immunoblot in AMA-negative PBC patients[125]. Recently, a study in Spanish PBC patients has confirmed the aforementioned data on the utility of these autoantibodies to diagnose AMA-negative patients by demonstrating anti-HK1 or anti-KLHL12 in a third of AMA negative and in 40% those negative for AMA, anti-gp210 and anti-sp100[127]. Furthermore, anti-HK1 positivity was associated with significantly higher possibility of liver decompensation and lower liver-related survival free of transplantation[127]. A more recent study assessed the prevalence of anti-KLHL12 and anti-HK-1 antibodies by ELISA at 5 sites within Europe and North America and documented the presence of these antibodies in patients with PBC in all geographies, irrespectively of the origin of the sera failing to identify geographic factors which could imply that the genetic make-up of the patients is responsible for the induction of these autoantibodies[128]. A Polish study reported the highest prevalence of anti-KLHL12 antibodies so far (36%) in both AMA-positive and AMA-negative patients and confirmed their high specificity for PBC diagnosis[110]. Their presence was linked to higher bilirubin levels and advanced fibrosis[110]. The prognostic significance of these autoantibodies needs to be evaluated in larger cohorts of PBC patients.

Bombaci *et al*[129] have proposed SPATA31A3 and GARP, as novel autoantigens in PBC, as PBC sera demonstrated high reactivity to these antigens irrespective of AMA and PBC-specific ANA presence [129]. Their combination with PDC-E2 assisted in discrimination of PBC from other diseases with high sensitivity and specificity. The authors offer in addition pathophysiological evidences on GARP expression in human cholangiocytes, which underlines the potential implication of this autoantigen in the induction of the disease[129].

MULTIPLEX ASSAYS

Considering that the repertoire of autoantibodies related to several autoimmune diseases is increasing, multiplex assays have become available in order to facilitate their simultaneous detection in a reliable, automated fashion, without consuming substantial amount of time. Such an example is that reported by Liu *et al*[130] in 2010 on a new PBC-screening assay that allows simultaneous detection of MIT3, sp100 and gp210 of IgG and IgA isotype by ELISA and compared these results with those from distinct IgG ELISAs[130]. Testing of 1175 PBC patients and 1232 non-PBC controls revealed sensitivity and specificity of PBC screen to be 83.8% and 94.7%, respectively, which is similar to that found from the evaluation of the combined data from individual MIT3, sp100, and gp210 IgG ELISAs[130].

Furthermore, assessment of 253 AMA-negative PBC patients by IIFL with this assay, resulted in positivity for PBC-specific autoantibodies in almost half of them (44.7%). Based on these data, PBC screen has been proposed as a reliable first line test for the diagnosis of PBC[130]. Comparable were the results from an Italian study, where examination of 100 AMA-negative PBC patients by IIFL showed reactivity in 43% of these patients with the use of the PBC screen[131].

Data from a study using a multiplex line blot assay, designed for autoimmune liver diseases, that contained AMA-M2, M2-E3 (a recombinant fusion protein including the E2 subunits of PDC, BCOADC and OGDC), as well sp100, PML and gp210 recombinant proteins had an overall sensitivity and specificity for PBC of 98.3% and 93.7% respectively. IIFL displayed lower sensitivity (86.2%), though comparable specificity (97.9%)[132]. Recently, a new automated PMAT assay on the Aptiva instrument, which includes MIT3, sp100, gp210, HK1 and KLp, facilitated the recognition of higher frequency of AMA- negative PBC patients compared to conventional immunoassays[57]. Even though multiplex technology seems to be a promising tool in the diagnosis of PBC, its use in the diagnostic algorithm of PBC needs to be further evaluated.

CONCLUSION

In conclusion, thorough discussion of the available literature reveals important aspects of the diagnostic, clinical and prognostic significance of disease-specific autoantibodies in AMA positive and AMA negative PBC patients[133]. Overall, identification of AMA and PBC-specific ANA is the cornerstone for the diagnosis of PBC. PBC-specific ANA, including anti-gp210 and anti-sp100, have a pivotal role in PBC diagnosis in AMA-negative individuals with high suspicion of the disease. Moreover, they have an established role as predictive factors for more advanced disease and worse outcome. The role of novel autoantibodies as diagnostic and prognostic tools in the management of patients with PBC needs to be assessed further.

FOOTNOTES

Author contributions: Rigopoulou EI had the original idea, wrote and revised the manuscript; Bogdanos DP wrote and revised the manuscript.

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MINIREVIEWS

Artificial intelligence as a noninvasive tool for pancreatic cancer prediction and diagnosis

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Abstract

Pancreatic cancer (PC) has a low incidence rate but a high mortality, with patients often in the advanced stage of the disease at the time of the first diagnosis. If detected, early neoplastic lesions are ideal for surgery, offering the best prognosis. Preneoplastic lesions of the pancreas include pancreatic intraepithelial neoplasia and mucinous cystic neoplasms, with intraductal papillary mucinous neoplasms being the most commonly diagnosed. Our study focused on predicting PC by identifying early signs using noninvasive techniques and artificial intelligence (AI). A systematic English literature search was conducted on the PubMed electronic database and other sources. We obtained a total of 97 studies on the subject of pancreatic neoplasms. The final number of articles included in our study was 44, 34 of which focused on the use of AI algorithms in the early diagnosis and prediction of pancreatic lesions. AI algorithms can facilitate diagnosis by analyzing massive amounts of data in a short period of time. Correlations can be made through AI algorithms by expanding image and electronic medical records databases, which can later be used as part of a screening program for the general population. AI-based screening models should involve a combination of biomarkers and medical and imaging data from different sources. This requires large numbers of resources, collaboration between medical practitioners, and investment in medical infrastructures.

Key Words: Pancreatic cancer; Early pancreatic lesions; Pancreatic neoplasia; Artificial intelligence; Deep learning; Machine learning; Radiomics; Diagnosis; Pancreas

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Core Tip: To improve the clinical management and prognosis for patients with pancreatic cancer (PC), new diagnostic methods should be developed to identify precursor lesions. Artificial intelligence (AI) is a tool that can offer a personalized approach in this regard by analyzing a large quantity of heterogeneous data and can also help in decision-making, increasing the prediction accuracy for an early diagnosis. The aim of this study was to provide a comprehensive overview of the advances in detecting PC noninvasively with an emphasis on early lesions and AI.

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INTRODUCTION

The World Health Organization estimates that early cancer detection will result in 30% greater cure rates for most cancer types[1]. Pancreatic cancer (PC) has a low incidence but is currently the 4th leading cause of all cancer deaths. The 5-year survival rate for PC is between 7% and 20%, with the best prognosis obtained for pancreatic neoplasms diagnosed in early stages[2-4]. Studies have reported that the 5-year survival rate can be as high as 39.9% in localized pancreatic tumors, but in patients who develop metastatic disease, the rate drops significantly to 2.9%[2-4].

There are two main types of PC, exocrine carcinomas and neuroendocrine neoplasms, with the former accounting for more than 95% of pancreatic tumors[5]. Patients with exocrine tumors compared with those with endocrine ones have a median survival of approximately 4 mo *vs* 27 mo[6]. Approximately 90% of exocrine PC cases are pancreatic ductal adenocarcinoma (PDAC); 80% of PDAC patients are in the advanced stage of the disease at the time of the first diagnosis[4,7]. A total of 49.6% of PDAC patients are diagnosed with distant metastases, and only approximately 15% have a surgically resectable tumor[1,2,4,7]. By 2030, PDAC is expected to become the second most common cause of cancer-related death in Western countries[8]. An important aspect in the diagnosis of PC is the size of the tumor, which correlates with outcome. For early-stage PDAC with node-negative, small tumors (< 2 cm), the survival rate can be as high as 80%[8]. Less than 2% of PCs are pancreatic neuroendocrine tumors (PNT). Current data have shown that an earlier diagnosis of PNTs is not associated with an improved survival rate. Compared with PDACs, PNTs have a better prognosis[9,10].

Preneoplastic lesions that frequently arise in the exocrine pancreas include pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN), and intraductal papillary mucinous neoplasms (IPMNs)[11]. Low-grade PanIN is quite common (present in 40%-75% of adults), especially after the age of 40[12]. The progression from low-grade PanIN to invasive tumors is unknown, and currently, identifying this type of lesion is not sufficient to justify surgical intervention. However, if high-grade PanIN is detected, surgery is highly indicated[1]. MCNs of the pancreas rarely progress to PC[11], while IPMNs are fairly common and, if left untreated, can progress to cancer[13]. An association between PDAC and IPMN has been reported in 11%-80% of cases[14]. Identifying high-risk IPMNs before surgical intervention enables patients in the low-risk category to avoid unnecessary surgery for benign disease. At this time, there are no accurate methods for the preoperative discrimination between high- and low-risk IPMNs[3,15].

Pancreatitis and cystic lesions of the pancreas are considered risk factors for developing PDAC. Chronic pancreatitis was identified in patients with PDAC 10 to 20 years earlier and acute pancreatitis 1 to 2 years before tumor diagnosis. Studies have shown that less than 5% of patients with chronic pancreatitis also develop PDAC. Cystic precursor lesions of PC include IPMN and MCNs, with IPMN being the most commonly diagnosed[1,14]. Pancreatic cyst fluid cytologic analysis is a candidate method for identifying progression to high-grade PanIN or cancer with a 25%-88% sensitivity in detecting pancreatic malignancy[1,16].

A challenge in the early diagnosis PC is the absence of specific symptoms or clinical data for patients with pancreatic tumors. The symptoms of PC, if present, are not specific and manifest approximately 6 mo after the identification of PDAC. Patients with symptoms from PC complain of abdominal pain and unexplained weight loss and are often found to have jaundice[4]. The majority of patients are asymptomatic, however[14].

Initial tests for evaluating patients suspected of having PC include serological investigation and abdominal imaging[5]. The latter is often performed using magnetic resonance imaging (MRI), MRI cholangiopancreatography, computed tomography (CT), endoscopic ultrasound (EUS), endoscopic

retrograde cholangiopancreatography (ERCP), and positron emission tomography[5,14,17]. One of the newest techniques in MRI involves diffusion-weighted imaging, which is capable of discriminating between PC and pancreatitis[17]. Certain features can be indicative of malignancy on imaging studies of early lesions for PC, including internal septation, mural nodularity, solid masses, duct dilatation, and vascular invasion. In 36%-70% of IPMN-associated cancers, mural nodules are diagnosed[14]. CT and MRI have a 56%-88% accuracy in detecting IPMN, but EUS has a higher resolution[14]. Pancreatic cysts are found in 3% of CT scans and 20% of MRI scans and can reach a 40% incidence in patients over 80 years old[14]. The most common imaging methods used, however, are EUS and CT. New EUS techniques (contrast-enhanced EUS and elastography) and the use of two or more imaging methods can enhance the diagnostic accuracy[7].

Additional methods implemented for PC include the analysis of personal data, biomarkers, and genomic features. Conditions such as family history of PC, body mass index, smoking, and alcohol abuse are epidemiologically associated with PC[18]. A total of 75%-80% of PCs are found in the head of the pancreas[14]. Diabetes is identified in 10%-40% of cases with IPMN, and a strong association between insulin and the risk of IPMN has been reported[14]. Patients with Peuts-Jegher syndrome, McCum-Albright syndrome, and familial adenomatous polyposis have also been diagnosed with IPMN [12,14]. MicroRNAs are biomarkers that have shown potential as a diagnostic tool for PDAC because of their high expression in the plasma of these patients[2]. MUC4, MUC16, differentially methylated DNA, telomerase protease expression, and overexpression of Das-1 biomarkers are associated with PDACs and are currently being validated for clinical use[1,14]. Elevated levels of carbohydrate antigen (CA)19-9 and carcinoembryonic antigen (CEA) and the identification of a new protein (cell migration-inducing hyaluronan-binding protein) are considered useful in PC prediction[14,19]. High concentrations of CEA are reported in mucinous pancreatic cysts but lack the potential to differentiate between IPMNs and PDACs[14]. The only biomarker for PDAC commonly considered reliable is CA19-9, but in the early stage disease, it has a sensitivity between 25% and 50% and is present in 51.1% of patients with PC stage I and 44.1% of patients with stage II[1,5].

Specific genomic features have been described for PDAC[1]. The top genes targeted during pancreatic carcinogenesis include KRAS, CDKN2A, TP53, and SMAD4. In patients with hereditary pancreatitis, mutation of the PRSS1 gene is associated with a higher risk of PC[12]. In early lesions of low-grade PanIN, KRAS mutations have identified, while inactivation of CDK2A and alterations in TP53, SMDA4, and BRCA2 occur in high-grade PanIN. Patients with these gene alterations can be classified as high-risk PDAC cohorts and considered for PC screening. These cohorts can also include patients with pancreatic cysts, pancreatitis, and new-onset diabetes who are 60 to 75 years of age[1]. KRAS, GNAS, TP53, SMAD4, PIK3CR, PTEN, and AKT1 mutations have been reported in IPMN-associated neoplasms with a 32%-89% sensitivity and 96%-100% specificity[14]. However, screening for PC asymptomatic subjects without a well-defined high-risk group is currently considered cost prohibitive[1].

Surgery is seen as a potentially curative treatment for PC if the patient is diagnosed in the early stage. For these patients, the pancreatic tumor can be completely resected, and by doing so, the survival rate significantly increases to 50%[4]. Treatment for the advanced state of PC typically includes partial resection, which is associated with tumor recurrence and a 5-year survival rate of less than 10%[4]. Unfortunately, pancreatic resection for PC has a high morbidity rate of up to 60%. Postoperative pancreatic fistula, stroke, cardiac arrest, wound dehiscence, infection, hemorrhage, and renal failure have been reported in patients the first month after pancreatic surgery[20]. Considering these factors, predicting PC by diagnosing early lesions and implementation of a screening program for PC becomes a matter of paramount importance. Artificial intelligence (AI) models integrating multisource risk factors are the future of early PC diagnosis.

Machine learning (ML), deep learning (DL) and the ability of computers to independently solve problems by using specific algorithms, is known today as AI. Although the terms ML and AI are commonly used interchangeably, ML is a subfield of AI. ML is based on developing mathematical algorithms for the analysis of data with the goal of creating a prediction model by recognizing patterns in these data. The algorithms can produce a machine-based diagnostic outcome for a specific disease. ML algorithms can be supervised or unsupervised and represented by support vector machines (SVMs), Bayesian interference, regressions, ensemble methods, decision trees, k-nearest neighbors, linear discriminants, and neural networks[16,21]. Supervised algorithms are based on data previously known by the computer, meaning that the computer is first trained with datasets from the domain of interest to later accurately analyze new datasets with the same subject and produce the desired mathematical outcome. In unsupervised algorithms, the computer sorts the input data and identifies features that are grouped into clusters or associations and analyzes them to reach a desired outcome[22]. Reinforcement learning is an advanced type of ML that is used to develop models that will help in the prediction of cancer but also facilitate the decision-making process[23]. Deep reinforcement learning is the combined process of reinforcement learning with DL. DL methods are trained to analyze large datasets through multilayer neural networks[24]. Developing algorithms by using AI methods helps in analyzing large amounts of data at the same time and integrating data obtained from different sources. Therefore, the results of clinical tests, imaging, personal data, biomarkers, and genomic features can be quickly analyzed, compared with existing knowledge, and used to reach an initial machine-made diagnosis. The purpose of our study was to identify the current diagnostic methods for detecting PC by using

noninvasive techniques with an emphasis on early lesions and AI.

METHODS

English-language literature published in the last 10 years covering diagnosing PC was searched by accessing the PubMed electronic database and other sources. The following key words were used for the search: "Pancreatic cancer", "early pancreatic lesions", "predicting pancreatic neoplasia", "artificial intelligence", "deep learning", "machine learning", "radiomics", "diagnosis", and "pancreas". We obtained a database of 97 studies on pancreatic neoplasms, including both single studies and reviews. We then removed duplicates, resulting in 86 papers. We excluded abstracts, reviews with insufficiently explained results, and articles not in the field of interest/irrelevant topics or with repetitive information. The final number of articles included was 44. The article selection algorithm for the study is depicted in Figure 1. We divided the remaining articles into two datasets. One contained the newest review articles and studies on obtaining information about current knowledge in assessing PC (10), and the other included studies that explored the use of AI algorithms in the diagnosis of pancreatic lesions (34). This second dataset included 24 studies on the current status of AI diagnostic methods for PC and 10 studies discussing the implications for early PC and prediction of the use of AI algorithms. The obtained data from the two datasets are presented narratively. The results of our analysis on the PC prediction methods for diagnosing early lesions are summarized in Table 1 and Figure 2.

BRIEF DESCRIPTION OF AI IN MEDICINE

ML in medicine is based on analyzing digital medical images from CT, MRI, and EUS and extracting image features in a process called radiomics. These radiomics features are converted into data that can be subjected to statistical analysis. In supervised methods, a human operator selects and labels the lesion in an image that will constitute the input data before an ML algorithm analyses these data. Unsupervised algorithms use a large amount of data for training and learn how to extract the region that will be later subjected to analysis. These mathematical algorithms will then examine the input data through a network similar to the neuronal network of the human brain. The human central nervous system receives input through various receptors, and then sends this information to the brain *via* the ascending pathway, where the data is then examined before a specific response is delivered through descending pathways to the intended receiver organs. There are similarities between the AI network and the human model of neural processing. We can consider humans as receptors of patient information, the input data as the ascending pathways, computers organized in layers as the brain, and output data as the descending pathways reaching the desired outcome as the receiver. Deep neural network or DL models are composed of sets of so-called hidden layers capable of studying a large amount of data. Each of the layers selects and amplifies certain features of the input information. For example, the first layer identifies a specific region and its length, the next layer analyzes the depth, another layer detects the pattern, and the subsequent layers compare the input with known or trained patterns so that when the information is clear, it is transformed into the output represented by the feature that best characterizes a specific type of lesion. In this way, AI is similar to humans but with significantly reduced human intervention and fewer human errors[25].

The interest in AI in healthcare is growing. The SVM algorithm has been used to classify breast cancer and has achieved a lower error rate than K-nearest neighbor, naïve Bayes, and C4.5 algorithms[22,26,27]. Electronic phenotyping algorithms have been built by researchers for predicting a disease correctly by using the electronic health records of the patients[22]. A convolutional neural network (CNN) has been used to prognosticate rectum toxicity in cervical cancer subjects with data provided from beam radiotherapy and brachytherapy. An alternative to surgery for the treatment of PC is radiation therapy. Given the challenging anatomy of the pancreas, sometimes the target area for treatment is difficult to identify. For these patients, metallic fiducials are implanted into the tumor target. These fiducials, however, can cause metal artifacts, obscure the tumor target, or be a source of complications such as pancreatitis or infections, all leading to delayed treatment. Therefore, developing mathematical algorithms for PC radiation therapy is a current subject of research. AI models using CT image data to localize the target PC for radiation therapy and calculate the dose of stereotactic body radiation have been reported with encouraging results[16,25]. DL algorithms have analyzed the mitotic rate of neoplastic cells to classify tumors. DL involves up to 150 hidden layers in the neural network, while traditional neural networks contain 2-3 layers. Some authors have suggested that tumors can be characterized by using computer aided design tools with supervised and unsupervised DL algorithms using CNN and transfer learning techniques. CNNs can be trained to automatically extract relevant features from images and classify the objects in the images with acceptable accuracy[22]. For AI-driven technologies to see further development in healthcare, ethical concerns must be addressed. Because AI algorithms require large datasets for training and for validation, data collection, storage, processing, confidentiality, and sharing must be considered[23].

Table 1 Studies exploring artificial intelligence algorithms for the diagnosis of early lesions and prediction of pancreatic cancer

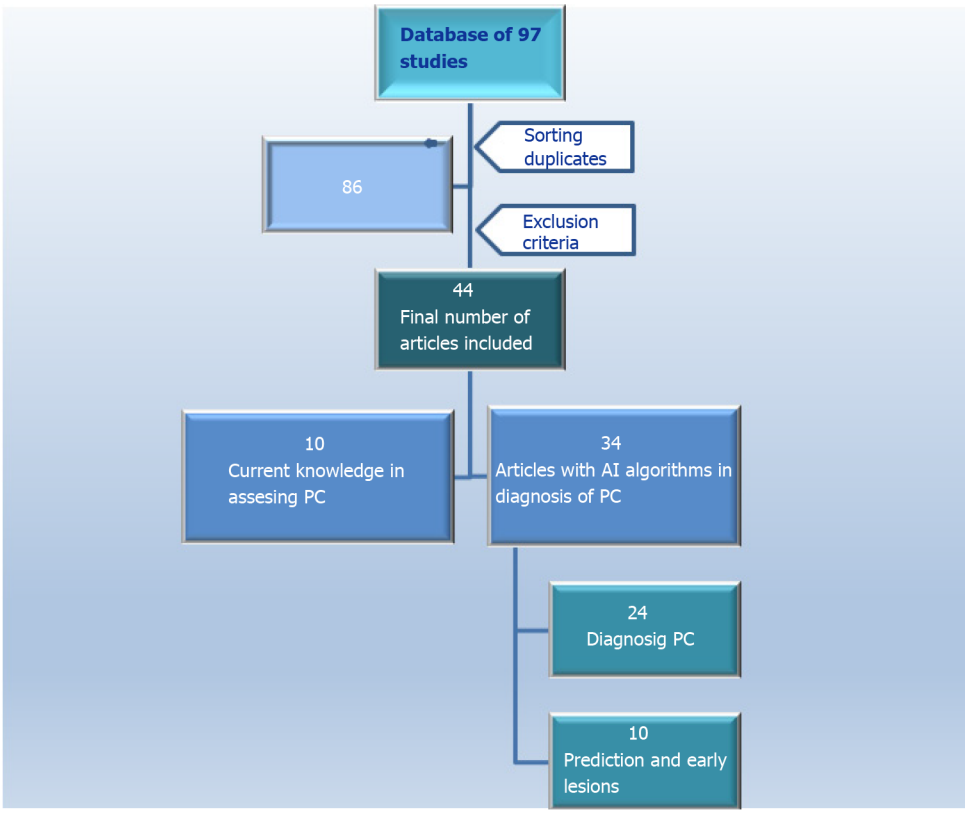
Ref.	Purpose of the model	Type of study	Type of model	Input data	Type of validation	No. cases
Qureshi *et al*[19], 2022	Identifying predictive features on prediagnostic CT scans for PDAC	Retrospective	NB	4000 radiomics from CT	External	72 (36 with PC)
Sekaran *et al*[22], 2020	Predicting PC	Retrospective	CNN	19000 images from CT	Internal	80 (NS)
Chen *et al*[36], 2018	Identification and classification methods for PC on MRI	Retrospective	CNN	863 images from MRI	Internal	40 (20 with PC)
Muhammad *et al*[18], 2019	Prediction of PC risk	Retrospective	CNN	18 features of epidemiologic and clinical data	External	800144 (898 with PC)
Alves *et al*[8], 2022	Detection and localization of small PDAC lesions on contrast-enhanced CT	Retrospective	DL	242 images from CT-CE	External	242 (119 with PC)
Kuwahara *et al*[35], 2019	Investigate the value of EUS in predicting malignancy in IPMN	Retrospective	CNN	3970 radiomics from EUS	Internal	50 (23 malignant)
Hussein *et al*[3], 2019	Identification of IPMN	Retrospective	CAD	171 MRI images	Internal	171 (133 IMPN)
Chakraborty *et al*[15], 2018	Identification of high-risk IPMN	Retrospective	SVM	103 CT images	Internal	103 (27 high-risk IMPN)
Liu *et al*[28], 2020	Classifying images as cancerous or noncancerous PC	Retrospective	CNN	21105 CT images	Internal and external	1242 (752 with PC)
Lee *et al*[44], 2022	Prediction of risk for PC	Retrospective	DNN	9 factors	Internal and external	2952 (738 with PC)

NB: Naïve Bayes; CT: Computed tomography; CNN: Convoluted neural network; PDAC: Pancreatic ductal adenocarcinoma; PC: Pancreatic cancer; NS: No specification; DL: Deep learning; EUS: Endoscopic ultrasound; IMPN: Intraductal papillary mucinous neoplasms; CAD: Computer-aided diagnosis; SVM: Support vector machine; MRI: Magnetic resonance imaging; DNN: Deep neural network.

NONINVASIVE METHODS FOR DIAGNOSING PC

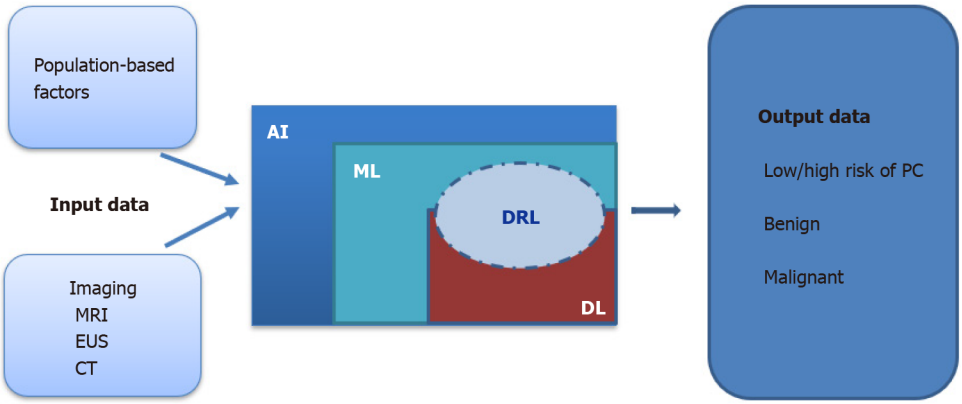
The potential of CNNs has been explored in the diagnosis of skin neoplasms, diabetic retinopathy, and liver tumors, but its utility in the detection of PC remains to be fully determined. CNN algorithms have been used for segmentation of the pancreas, risk stratification for IPMN, assessment of the grade (G) of pancreatic neuroendocrine neoplasm, targeting PC for radiotherapy, and classification of the pancreatic cysts[28].

In the diagnosis of PCs, AI is used for the analysis of radiomics features from CT, MRI, and EUS, of images from histopathological slides, and of tumor markers[29] (Figure 3). Because CT is commonly performed when investigating patients, it is currently the most explored imaging modality with AI. In the United States, approximately 7 million patients per year present to the emergency room with abdominal pain, for which a CT scan analysis is required by hospital protocols. These protocols offer a database that can be consulted for patients who later develop PC[1,19]. In identifying PC, contrast-enhanced CT has a sensitivity of 70%-100%, and the usual CT scan has an accuracy of 83.3%, sensitivity of 81.4%, and specificity of 43%. In the detection of PDAC, CT has a sensitivity of 76%-96% and offers information about the location, size, and morphology of the pancreatic mass[1,2,25]. According to Qureshi *et al*[19], healthy cells transforming into neoplastic cells appear darker on a CT scan. Even with these results, CT scan-based analysis is not taken into consideration as a screening method for early pancreatic lesions because of the constant exposure of the patient to radiation[1,2,25]. MRI techniques identify early modifications in the pancreatic parenchyma, such as fibrosis and inflammation, with enhanced resolution. New developments in these techniques have allowed the evaluation of tissue vascularization[3]. PDAC can be diagnosed on MRI with an accuracy of 89.1%, sensitivity of 89.5%, and specificity of 63.4%. EUS has demonstrated the highest precision in tumor detection for pancreatic lesions, with a sensitivity as high as 74%-94%[1,25]; in the detection of PCs, an endoscopist analyzing EUS images can reach a sensitivity of 94%[1,29-31]. Some studies have shown that even though PDAC is the most commonly identified histopathological type of PC, PNTs have a higher predominance in small pancreatic lesions. The differential diagnosis between PNTs and other types of PC can be made using EUS because of the better contrast offered by their rich vascularization[30]. In neuroendocrine tumors,



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Figure 1 Article selection algorithm for the study. A total of 97 studies and reviews were identified with key words; 86 papers remained after removing duplicates; 34 eligible studies were finally included: 24 on the current status of artificial intelligence (AI) diagnostic methods for pancreatic cancer (PC) and 10 discussing the implications of AI algorithms in early PC and prediction. AI: Artificial intelligence; PC: Pancreatic cancer.

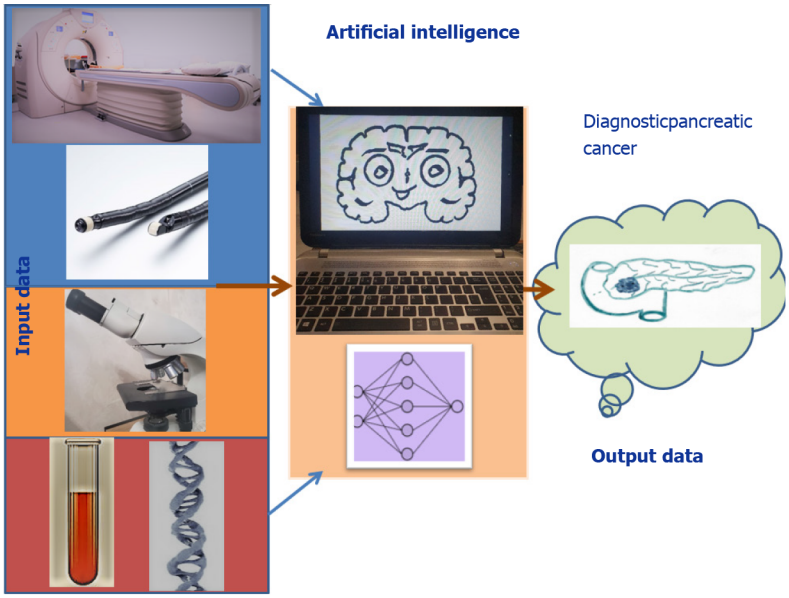


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Figure 2 Pancreatic cancer prediction methods for diagnosing early lesions. AI: Artificial intelligence; ML: Machine learning; DL: Deep learning; DRL: Deep reinforcement learning; PC: Pancreatic cancer; CT: Computed tomography; EUS: Endoscopic ultrasound; MRI: Magnetic resonance imaging.

EUS has a 75%-97% detection rate for PNTs but gives the best results when combined with MRI studies in the initial assessment[30]. By using AI algorithms in EUS for detecting PC, the sensitivity increases to 83%-100% with an overall accuracy of 80%-97%[1,21]. However, there are limitations to using EUS as a common analytical method for clinical diagnosis; although it is more cost-effective than MRI, EUS is limited by: (1) The need for patient sedation; (2) The need for a technician with suitable knowledge and experience in performing the method; and (3) The narrow field investigated, as EUS cannot visualize extrapancreatic organs[1]. The sensitivity of EUS in diagnosing PC ranges from 54%-98%; hence, new techniques, such as ML algorithms, are needed[16,25].

Diagnosing PC is often difficult due to the lack of well-defined radiographic images and symptoms of the disease in early lesions as well as mimickers of neoplasia[4]. In performing CT segmentation, a radiologist must analyze approximately 300 images containing visual information for each patient, so a



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Figure 3 Artificial intelligence leading concepts for pancreatic cancer diagnosis. Input data: Imaging, endoscopic, and histopathologic data and tumor markers; artificial intelligence with machine learning and deep learning; output data: Diagnosing pancreatic cancer.

mathematical algorithm developed for the analysis of data extracted from a CT scan of the patients listed as high-risk in a screening program would increase efficiency[1]. Even though the image of a neoplasm can be identified months before the time of diagnosis, the sensitivity for PC is low, so a screening method involving AI algorithms could help predict and identify early stages of PC. On CT scans, PC presents with irregular contours and poorly defined margins. Early lesion studies have shown that on CT, alterations are present in the pancreas 18 mo before the diagnosis of PDAC but with a sensitivity of about 15%[1,2,25]. In early disease stages, when the lesions are small, these masses can be overlooked by radiologists[31]. Typically, pancreatic tumors smaller than 2 cm are difficult to identify from the surrounding tissue, and the gland itself represents approximately 1.3% of a CT image, offering only a small amount of data[1,2,31]. Lesions that can alter the depiction of the pancreas on a CT scan by causing shape and size deformations and that appear similar to PC include parenchymal atrophy, IPMN, lithiasis, pancreatitis, and pancreatic duct ectasia. These lesions increase the tissue heterogeneity of the pancreas on CT, making the diagnosis of PC difficult[4]. One report showed that in 90% of cases, the misdiagnosis of PC on imaging was due to inflammation that obscured the underlying tumor mass [23]. Studies of PC with AI have been conducted to help classify PNTs by analyzing preoperative CT and MRI accurately and showed that these AI algorithms can be useful in differentiating exocrine from endocrine PC in complicated cases. Commonly, there are different patterns in the vascularization of PNT and pancreatic adenocarcinomas, but there are small proportions of cases with atypical findings that make the differential diagnosis difficult, and for these situations, a histogram analysis on CT can be used[31]. Studies have assessed tumor grade, with results showing that DL methods applied to MRI can differentiate between histological aspects of PNT by classifying G1 from G2 and G3 neoplasms and G1 and G2 from G3[31,32].

AI algorithms for EUS, MRI, and CT images in PC diagnosis

EUS has an elevated detection rate for IPMN and *in situ* PC (stage 0)[33]. Digital image analysis of EUS images using computers with SVM- and artificial neural network (ANN)-based methods has demonstrated high accuracy[25,34]. The overall accuracy in using ANNs on EUS for the diagnosis of PC ranges between 89%-94%. Elastography performed during EUS is reported to have an accuracy of 83%-90%; this imaging technique can differentiate the consistency of the issue on an EUS image by color coding, with red indicating softer tissue and blue indicating harder areas[25]. Better results of EUS analysis with AI techniques have been obtained by studying an extended neural network of EUS elastography images with an accuracy of 89.7% in differentiating between malignant and benign pancreatic lesions. Additionally, contrast-enhanced EUS and contrast-enhanced harmonic EUS have been shown to be useful in identifying focal pancreatic masses together with a diagnosis of a benign or malignant lesion. In contrast-enhanced EUS, contrast agents are intravenously injected for better identification of focal pancreatic lesions[21]. Preoperative AI sorting of EUS images of IPMNs presented a higher accuracy in predicting the malignant component than a human technician (94% *vs* 56%, respectively). A study performed with a DL back propagation master for EUS training and quality control, which had both internal and external validation, achieved an accuracy ranging from 82.4% to

94.2% in detecting PC. The decision tree method, implemented for patients who underwent ERCP, precisely identified PC with an 87%-91% sensitivity and 80% specificity[16].

A meta-analysis of published data aiming to investigate the diagnostic value of AI EUS in PC showed a very good diagnostic accuracy. The meta-analysis extracted data from 10 studies that included 1871 patients with AI algorithms based on a CNN, ANN, and SVM. The ANN model had the best accuracy in detecting PC, with a sensitivity in detecting small pancreatic tumors of 93% for EUS, 53% for CT, and 67% for MRI. However, the authors stated that the results of these 10 studies did not have a general utility because the data were evaluated only with internal validation, and thus the results could have been overestimated. To be relevant for patients from different populations, the sources used for validation must be diverse, external, and from more than one center[24]. Studies have reported that ML models such as ANNs and CNNs can be trained to extract quantitative data that can be correlated with the histological type of PC but also with the survival rates and the response to chemotherapy[11,25,31].

A retrospective study was performed by Kuwahara *et al*[35] on 50 patients diagnosed surgically with IPMN. A total of 3970 radiomics features from EUS were investigated with a CNN algorithm. After augmenting the data, the sample fully investigated was composed of 508160 images. The author's goal was then to classify IPMNs as benign or malignant. For this, the ability to predict early lesions was investigated by comparing AI methods with physicians. The AI methods demonstrated an accuracy of 94.0% in predicting malignancy, while the humans achieved an accuracy of 56.0%. This study had a number of limitations; however, it was retrospective, had a small sample of cases, only performed internal validation, and used data from only one center. Additionally, all the patients included in the study were surgically investigated, and for IPMNs, the recommendation was surveillance[35].

Chen *et al*[36] implemented a number of 3D CNNs, including ResNet18, ResNet34, ResNet52, and Inception-ResNet, to classify pancreatic tumors from MRI images. These authors used the MRI images of 20 normal patients and 20 patients with tumors, from whom 77 benign MRI images and 38 malignant MRI images were obtained, and through data augmentation, 442 benign MRI images and 421 malignant MRI images were finally analyzed[36]. Data augmentation oversamples images to generate synthetic data in cases with small sample sizes and limited availability of image data, such as rare cancers and PCs[31]. The ResNet18 method achieved an accuracy of 91% in classifying benign and malignant lesions. Other studies have reported analyses with 3 DCNN using MRI for Alzheimer's disease diagnosis and the classification of lung, brain, and prostate lesions[25,31,36].

Hussein *et al*[3] used the MRI data from 171 subjects to identify IPMN, including 38 normal subjects and 133 diagnosed with IPMN. Two-dimensional axial slices were used to generate regions of interest, and supervised and unsupervised learning was used in computer-aided diagnosis. The authors used unsupervised DL features for image classification with a CNN, and the highest accuracy in classifying the IPMN was obtained with nonlinearity clustering and implementation of a VGG-fc7 layer. In supervised learning, the deep network associated with adaptive synthetic sampling performed better on small datasets[3].

Chakraborty *et al*[15] studied CT images from 103 patients confirmed with IPMN using SVM and random forest methods. The random forest algorithm had better results in extracting the features of the IPMN lesions. These authors experimentally developed an architecture designed for predicting high-risk patients with IPMN, but their method lacked independent validation. Additional limitations include its retrospective nature, the need for manual segmentation of the lesion and the pancreas, and the use of small datasets. Pancreatic cystic lesions were identified on 2.6% of the abdominal CT scans, with 25% of all lesions being diagnosed as IPMN and approximately one-third being associated with invasive carcinoma. The criteria for establishing whether IPMNs are at low or high risk of harboring malignancy are limited. Distinguishing between these two lesions is important, as low-risk patients are recommended to undergo surveillance, while high-risk patients should undergo surgical resection[1,16].

Alves *et al*[8] performed a study on the contrast-enhanced CT scans from 119 PDAC patients and 123 patients without PDAC. PDAC was detected using a self-configuring framework for medical segmentation, NNU net, focusing on small lesions and assigning a label of tumor or nontumor. Their results showed that DL models can diagnose early PDAC lesions, but the study included only tumors of the pancreatic head and required resources for the manual labeling of PDAC images[8].

In an attempt to use AI as a prediction model for early PDAC diagnosis, Qureshi *et al*[4] used three types of radiomics features from CT scans. Two types belonged to the same patients but were executed (one before and one after the diagnosis was histopathologically confirmed) and another type was extracted from normal subjects. The prediagnostic CT scans of the patients were obtained 6 mo to 3 years before the PC was identified. In their study, Qureshi *et al*[4] analyzed 108 CT scans from 72 patients classified into two datasets, an internal dataset consisting of 66 contrast-enhanced abdominal CT scans for building the model, and an external dataset consisting of 42 scans for validation. A naïve Bayes classifier was trained to perform automatic classification of the CT scans of two groups, one representing the healthy control group and the other the prediagnostic group. A total of 4000 radiomics features were extracted from each of the 66 scans with the aim of identifying patterns in the images obtained before and after the PC diagnosis. The study concluded that prediagnostic CT images provided sufficient information to validate the contribution of AI in predicting patients at risk of PDAC, but further studies are needed to address the potential overfitting due to the limited dataset used[4].

A retrospective study on 6084 contrast-enhanced CT images in patients with histopathologically confirmed PDAC used a Faster R-CNN model to develop an automatic system for automatically diagnosing PC. The AI produced its diagnoses in 3 s, shorter than the 8 min required by the image specialist. The limitations of this study were as follows: It was a retrospective study of patients with PC diagnosis pathologically confirmed from a single center, normal patients and those with benign lesions were excluded, and it did not have external validation[37].

Sekaran *et al*[22] proposed a DL network by analyzing a dataset consisting of 19000 radiomics features from 82 abdominal CT images. The method involved a Gaussian mixture model with an EM algorithm and a DL CNN. They proposed a lump recognition algorithm, with the input or region of interest being the area in which the growth of a lump is detected. Each layer of the CNN extracts features of this region to build a model for assessing the features of the lump in terms of size, shape, and weight. Their results were able to help the patients by identifying the rate of spread of the tumor in the head of the pancreas after diagnosis and treatment. This study was limited by the fact that the tumors were analyzed in only one part of the pancreas, the head.

Liu *et al*[28] used two datasets, one consisting of 471 patients, 355 with histopathologically diagnosed or cytologically confirmed PC, and the other for external and internal validation. The control set participants had no pancreatic lesions at the time when the CT scan was performed. The study was conducted in the patch and patient levels. In the patch-based analysis, the region of interest was processed into patches, and the patients were classified as having or not having PC using a modified CNN model from the Visual Geometry Group. For the patient-based approach, age, sex, and tumor stage and size were taken into consideration. This study designed a CNN-based algorithm for classifying patients with or without PC by using CT scans, with an accuracy of 99%. Additionally, their method ensured avoidance of overfitting by using training and validation datasets that were sufficiently different but included patients of different races and ethnicities, preprocessing CT images into patches, and using data augmentation methods such as moving windows and flipping. Radiologists were provided with the clinical data of the PC patients, and the CNN had no information. The patches used for training the CNN were segmented by the radiologists. The limitations of these results were manual labeling of pancreatic images with a modest sample size and only Asian participants from a single institution.

Cardobi *et al*[38] found that a CNN-based analysis on CT images for identifying malignancies in IPMNs showed a sensitivity, specificity, and accuracy in the classification of tumors of 95.7%, 92.6%, and 94.0%, respectively. Additionally, AI algorithm models built for the segmentation of the pancreas and used to determine the pancreas volume in autoimmune pancreatitis were 2.38 times faster than manual approaches[38].

AI algorithms for diagnosing PC using tumor markers, images from histopathological slides, and epidemiologic data

Studies using neural networks for the analysis of tumor markers for PC diagnosis have shown that the diagnostic performance of a single marker is lower than that of the AI model multiple for tumor marker analysis. Detecting the values of only one of CA19-9, CEA, and CA125 for PC diagnosis with AI has a low sensitivity[39]. In a mouse model, Serrao *et al*[39] measured the concentrations of alanine and lactate and the activities of lactate dehydrogenase and alanine aminotransferase in the pancreas of animals with different lesions ranging from pancreatitis to tumors. Their method used metabolic magnetic resonance spectroscopic imaging with hyperpolarized [1-^{13}C] pyruvate for detecting and monitoring the progression of PC precursor lesions. Their results were able to distinguish pancreases with predominantly low-grade PanIN from tissue with high-grade PanIN and tumors. However, the studied animals had a small pancreas, and the high-grade PanINs occupied approximately 40% of the tissue. The human pancreas is larger than that of mice, and these lesions are usually small, so additional data are needed before this method can be used for human patients[18,32]. A new field of liquid biopsies offers data available from biomarkers such as exosomes, proteomes, proteins, cell-free DNA, and circulating microRNA that can be analyzed using AI methods for the early diagnosis of PC[24].

With the development of ML, digital medical imaging can play a role in assisting with diagnosis based on histopathological images. Today, it is possible to digitize glass slides using whole-slide imaging. AI techniques have been applied in pathology slide analysis for prostate and breast cancers. For these methods, a high-resolution digital image of the tissue from the glass slide is obtained by using a specialized scanner; the images are then analyzed by AI methods. Currently, however, only a few studies have investigated the use of AI on physical PC specimens. With an ANN algorithm, an overall accuracy of 77% was obtained in the reclassification of specimens as malignant or benign[25]. A study using a deep CNN on histopathological images of PDAC achieved a 95.3% classification accuracy by examining a total of 231 tissue samples from 171 PC and 60 normal pancreas slides using whole-slide imaging and image augmentation techniques[40]. Sehmi *et al*[41] developed DL models for grading PC from pathology slides that had a 95.61% classification accuracy. Naito *et al*[42] trained a DL method to assess PDAC on endoscopic ultrasonography-guided fine-needle biopsies and obtained a model that was able to detect small amounts of cancer cells in difficult cases. Cutting-edge digital pathology tools that can scan, analyze, and store data from an entire tissue sample continue to be developed[23].

Si *et al*[43] proposed a fully end-to-end deep-learning-based (FEE-DL) algorithm for the automatic diagnosis of different types of PCs. A total of 284 PCs, such as IPMN, PNET, serous cystic neoplasms, and a group labeled "other"-represented by gallbladder tumors, cholangiocarcinoma, ampullary carcinomas, duodenal cancer and metastasis-was investigated. The dataset was augmented using random elastic brightness, random contrast, random elastic transformation, and random cropping. The FEE-DL consisted of a tree connected subnetwork: ResNet18 for recognizing images containing the pancreas, U-Net-32 for making predictions on each image for pancreas segmentation, and ResNet34 for diagnosing pancreatic tumors. This model had an average accuracy of 82.7% for all tumor types, with a 100% accuracy in identifying IPMN and 87.6% for PDAC on a dataset that included 347 patients following four stages: Image screening, pancreas location, pancreas segmentation, and PC diagnosis. The FEE-DL algorithm independently identified different histologic types of PC with precise results in detecting PDAC and IPMN.

Muhammad *et al*[18] designed an ANN method to predict PC based on a study of a total of 18 personal health features (age, diabetes, smoking, exercise status, alcohol consumption, and family history of PC) from 800114 participants, including 898 with PC. Their results showed that the risk of PC can be predicted and stratified using an ANN that analyzes easily obtainable personal health features with an 80.7% sensitivity and 80.7% specificity. This study used two broad data sources: The National Health Interview Survey, established in the United States to monitor the general health status of the population with 131 PC patients from 645217 respondents, and the Prostate, Lung, Colorectal and Ovarian screening program, including 797 PC patients from 154897 participants[18].

Lee *et al*[44] developed a CNN predictive model for PC using data from the Taiwan Health Insurance database, which covers 99.98% of the population of the region. A total of 3690 subjects were selected, 2952 of whom presented with risk factors associated with PC, including pancreatitis, diabetes, peptic ulcer, cholangitis, hepatitis, periodontal disease, sleep disorders, and fasciitis, but factors obtained from the subjects' medical history were further added. Finally, 74 candidate factors were included in this study; 738 patients had PC, and 2214 subjects representing the control group were cancer-free. This study constructed a PC prediction model with an accuracy higher than that of previous studies using nine key independent predictors: Abdominal pain, peptic ulcer, flatulence, gastritis, abnormal gastric function, hepatitis, sleep disorders, cholangitis, and pancreatitis.

FUTURE PERSPECTIVES

For PC, a cost-effective screening method for early lesions is needed. AI models developed for a diverse group of patients from high-risk PDAC cohorts belonging to broad datasets from different sources can be of great use for improving PDAC treatment outcomes and enhancing diagnostic precision. AI methods represent a needed step to reach a standardized interpretation of patient data and investigations while reducing human bias or error. For this, a wide range of data from different sources is necessary. All of these data have a central role in training AI methods in diagnosing PC and establishing the most accurate algorithm to be used for such a diagnosis. Correlations can be made through AI algorithms by expanding the image and electronic medical record databases, which will significantly improve the diagnostic accuracy and provide an early diagnosis for PC patients, thereby producing a better prognosis and more effective therapy. AI creates a common ground in diagnosing PC that can be standardized and used for the general population. Such an AI model should contain a combination of biomarkers, medical data, and imaging data obtained by alternating CT, EUS, and MRI testing so that maximized accuracy and early diagnosis with noninvasive techniques can be achieved.

The coronavirus disease 2019 pandemic has produced a model for forming an international infrastructure that can be studied with AI algorithms and even used for predicting and early diagnosing cancers. The collaboration between scientists and academic centers has shown that humans from different countries and continents can work together to share information in a common attempt to save lives while creating a vast database. This will require resources, global solidarity, and support investment in medical infrastructures worldwide. However, the need to develop health infrastructure and multidisciplinary research studies is crucial, as the pandemic situation has already shown.

CONCLUSION

Our analysis of the current diagnostic methods for detecting PC noninvasively with an emphasis on early lesions has revealed a series of common features and limitations: Published studies about predicting early lesions in PC are lacking. For PC diagnosis, noninvasive tests such as imaging, tumor marker analysis, and population-based studies can be used to train AI algorithms to identify features, patterns, and subtle changes that can help classify pancreatic lesions and build predictive models that can improve the awareness of the risk of pancreatic neoplasia. Published studies typically included only small samples from a limited number of cases. The analysis of these studies focused mostly on just one type of investigation (radiomics features, personal data, biomarkers, or genomic features without

making correlations between all the data that a physician analyses for a diagnosis of PC in a specific patient). A high percentage of the studies only performed internal validation, so bias and overfitting can constitute a problem when generalizing their conclusions. Commonly, for machine diagnosis, the mechanism that generates the output is not clearly explained, so assessing how a specific AI algorithm makes its final diagnosis remains controversial. AI algorithms for assessing pancreatic volumes with and without tumors are time- and cost-consuming that are usually run manually and in direct relation with the radiologist's experience. Finding an AI-driven automated volumetric segmentation algorithm is difficult because of the variations in shape, the shallowness of the boundaries, and the small size of the pancreas. Pancreatic tumors smaller than 2 cm frequently have inconspicuous borders with high similarity to the surrounding tissues on radiomics analysis, making early lesion diagnosis challenging. However, AI methods have the potential to improve accuracy and support clinical decisions for the preoperative diagnosis of pancreatic lesions and to aid in the surgical management and prognosis of PC.

FOOTNOTES

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MINIREVIEWS

New uses for an old remedy: Digoxin as a potential treatment for steatohepatitis and other disorders

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Abstract

Repurposing of the widely available and relatively cheap generic cardiac glycoside digoxin for non-cardiac indications could have a wide-ranging impact on the global burden of several diseases. Over the past several years, there have been significant advances in the study of digoxin pharmacology and its potential non-cardiac clinical applications, including anti-inflammatory, antineoplastic, metabolic, and antimicrobial use. Digoxin holds promise in the treatment of gastrointestinal disease, including nonalcoholic steatohepatitis and alcohol-associated steatohepatitis as well as in obesity, cancer, and treatment of viral infections, among other conditions. In this review, we provide a summary of the clinical uses of digoxin to date and discuss recent research on its emerging applications.

Key Words: Digoxin; Cardiac glycosides; Oxidative stress; Nonalcoholic steatohepatitis; Alcohol-associated steatohepatitis; Sterile inflammation

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Core Tip: Digoxin has been used primarily as a cardiac drug for treatment of arrhythmias and heart failure. Preclinical work supports the repurposing of digoxin as therapy for non-cardiac conditions, including alcohol-associated steatohepatitis, nonalcoholic steatohepatitis, obesity and metabolic disorders, autoimmune and inflammatory conditions, malignancy, and viral infections, among others. Here, we provide an overview of findings to date on the potential clinical applications of digoxin and mechanisms of action in steatohepatitis and other non-cardiac disorders. We discuss evidence on the differential action of digoxin at high *vs* low concentrations and identify areas of further research necessary to harness its promising multifunctional use.

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INTRODUCTION

Digoxin in a nutshell: An overview of 200 years

Digoxin (also known by the broader term digitalis) is derived from the purple foxglove, a medicinal plant that can be traced to Irish monks and Germans and was cultivated during the time of Charles the Great (700s–800s). Its Latin scientific name *Digitalis purpurea* was coined by Leonard Fuchs in 1542 based on the translation of the German word describing the shape of the flower as a fingerhut or thimble. Digitalis was mentioned in herbal remedies in England in the 1500s and 1600s for several purposes, including epilepsy, vertigo, swelling/fluid accumulation, tuberculosis, and skin diseases[1]. Subsequently, digitalis fell out of favor due to reports of its toxicity. Animal experiments involving the administration of digoxin leaves to turkeys and roosters resulted in fits and death[1].

In the late 1700s, Withering[2], an English botanist and physician, heard about a family recipe containing over twenty different herbs used in the cure of fluid overload, referred to as dropsy[2]. After realizing that the active ingredient in the herbal remedy was likely from the foxglove plant, Withering [2] administered foxglove tea as a cure to a patient with dropsy. That patient did well, and over the ensuing decade he performed a comprehensive case series of digitalis by administering a decoction prepared from dried foxglove leaves to 163 patients with fluid retention, of whom 101 experienced relief. He noted that digitalis was especially helpful for patients with dropsy after having scarlet fever or bad sore throats. Withering's work inspired other physicians to try digitalis as a therapy in dropsy[2]. For further information on the evolution of digoxin as a medical therapy, the reader is referred to an excellent review by Wray *et al*[1].

The molecular formula of digoxin is $C_{41}H_{64}O_{14}$, and its molecular weight is 780.9 g/mol. Similar to other cardiac glycosides (CG), digoxin increases the force of contraction of the heart by reversibly inhibiting the activity of the myocardial Na-K ATPase pump, an enzyme that controls the movement of potassium ions into the heart[3-5]. The most common cardiac uses of digoxin include heart failure and supraventricular arrhythmia. Its role in heart failure is due to its inotropic properties, inhibiting the Na-K ATPase pump thus increasing the intercellular calcium concentration[6]. This lengthens the cardiac action potential, lowering the heart rate and increasing myocardial contractility. The American College of Cardiology/American Heart Association guidelines recommend that digoxin be added to the heart failure medication regimen in patients with left ventricular systolic dysfunction when symptoms persist despite optimization of treatment with an angiotensin-converting enzyme inhibitor, a β-blocker, and/or a diuretic[7-9]. The digoxin effect in treatment of supraventricular arrhythmia occurs through its parasympathomimetic stimulation *via* the vagus nerve, reducing automaticity of the sinoatrial node and slowing atrioventricular conduction[10].

Current clinical use of digoxin is limited to the cardiac arena. Oral digoxin is available as a solution (0.05 mg/mL) or as tablets (0.0625 mg, 0.125 mg, 0.1875 mg, and 0.25 mg). Dosing is typically maintained between 0.125 to 0.25 mg daily, with lower doses considered in patients 70 years of age or older[11]. The steady-state volume of distribution of digoxin is decreased in chronic renal failure; therefore, both loading and maintenance dosing should be decreased in such patients[12]. Digoxin has a narrow therapeutic window, with the rate of toxicity increasing as serum concentration reaches over 2.0 ng/mL. However, toxicity can also occur at levels below 2.0 ng/mL in the setting of risk factors such as age, decreased renal function, hypokalemia or other electrolyte abnormalities, or interacting medications[13]. The narrow therapeutic window of digoxin necessitates monitoring of serum digoxin levels, particularly in patients with chronic renal dysfunction or varying renal function.

With the discovery of many effective cardiac drugs for heart failure and supraventricular arrhythmias over the past few decades and difficulty maintaining the narrow digoxin therapeutic index, the use of digoxin in cardiac disease has been waning. During this period, however, there have been several

advances in basic and preclinical work toward the potential repurposing of digoxin and other CGs for non-cardiac conditions. These studies indicate that the biological effects of CG are not limited to the inhibition of Na, K-ATPase but include various signal transduction pathways including nuclear receptors (NRs) involved in hormonal signaling, immune response, and carcinogenesis, among others [14-19].

DIGOXIN IN STEATOHEPATITIS

Overnutrition and obesity impair metabolic homeostasis and trigger sterile-type inflammation[20-23], contributing to the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). The amplitude of sterile inflammation triggered by metabolic stress in the liver has major clinical consequences. Sterile inflammation is responsible for increasing amounts of liver damage and cell death in NASH[24]. NASH, as well as other diseases associated with sterile inflammation of the liver, lacks effective treatments. This is due to the relatively poor understanding of the initiating steps in sterile inflammation and the dysregulation of a wide range of pathways, making it difficult to know which ones to target.

Identification of hypoxia-inducible factor 1-alpha (HIF-1α) pathway activation in macrophages for sustained inflammatory responses provided HIF-1α with a key role in the core regulatory machinery for the transition from acute self-limiting to sustained chronic inflammation[25]. These mechanistic insights into the role of the HIF-1α pathway in sterile inflammation may have great clinical relevance due to the ability of digoxin to inhibit HIF-1α activation[26]. Digoxin (1.0-0.05 mg/kg) effectively prevents acute and chronic hepatic damage, steatosis, and inflammation in both lipopolysaccharide- and high-fat diet-driven animal models[27].

Digoxin reduces oxidative stress during liver injury by maintaining cellular redox homeostasis and protects the liver from a wide variety of insults[27,28]. Digoxin reduces HIF-1α transcriptional activity, thus disrupting HIF-1α-mediated antioxidant pathways. Digoxin induced significant changes in gene transcripts related to HIF-1α in metabolic processes and nucleic acid binding[27]. To understand the direct molecular mechanisms responsible for the digoxin effect on HIF-1α transcription, pyruvate kinase M2 (PKM2) was identified as the major digoxin binding protein using a novel approach of digoxin-immunized agarose beads coupled with liquid chromatography with tandem mass spectrometry analysis[27]. The ability of digoxin to bind to PKM2 was an unexpected finding and provided novel insights into PKM2 biology and the role of PKM2 in sterile inflammatory liver diseases. PKM2 is best known as the rate-limiting glycolytic enzyme that catalyzes the conversion of phosphoenol pyruvate and adenosine diphosphate to pyruvate and ATP[29].

In addition to its pyruvate kinase function, PKM2 interacts with HIF-1α in the nucleus and functions as a transcriptional coactivator for HIF-1α, resulting in the stimulation of HIF-1α responsive genes[30]. Interestingly, the interaction of digoxin with PKM2 did not alter its pyruvate kinase ability or reduce its nuclear translocation. Digoxin, however, reduced the ability of PKM2 to upregulate the transcription of HIF-1α and its downstream genes, such as inflammatory genes and genes involved in oxidative stress (Figure 1). Further, digoxin reduced the binding of PKM2 to histones, suggesting that digoxin suppressed PKM2-mediated transactivation of HIF-1α through chromatin modifications[27].

Oral digoxin significantly reduced high-fat diet-induced hepatic damage, steatosis, and liver inflammation across a wide dosage range[27]. The lowest dose of digoxin (0.125 mg/kg) showed significant protective effects against liver injury and sterile inflammation. Interestingly, digoxin had direct effects on the inhibition of inflammasome activation. Digoxin had a small effect on typical inflammasome activity while strongly inhibiting the HIF-1α pathway-sustained inflammasome activity in macrophages. Despite the importance of PKM2-HIF1α pathway activation in immune cells during NASH development [27], its direct effect on hepatocytes was unclear. PKM2 levels in healthy human liver cells were very low, but they were significantly elevated in NAFLD and NASH. Pyruvate kinase L/R, the major isoform of pyruvate kinase in the liver, was unchanged. Digoxin treatment directly inhibited PKM2 transactivation leading to the improvement of hepatocyte mitochondrial dysfunction, steatosis, and hepatocellular injury in the obese mouse model (Table 1).

NRs are ligand-activated transcription factors that are involved in a wide array of physiological processes. These transcription factors typically have different domains responsible for ligand-independent interactions with corepressors and coactivators, recognition and binding of response elements within target genes, interaction with other proteins or facilitation of protein translocation, as well as ligand-dependent functions[31-37]. The involvement of NRs in the regulation of a variety of metabolic and physiological processes makes them interesting pharmacological targets.

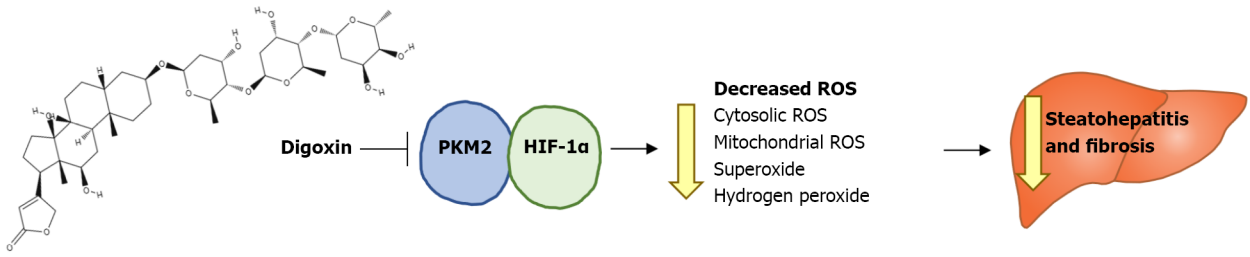
The NR gene retinoic acid-related orphan receptor C gene encodes two protein products, the retinoid-related orphan receptor-gamma (RORγ) and RORγT isoforms, which differ by 21 amino acids in their N-terminal A/B domains. The RORγ isoform is broadly expressed[38] and is involved in the regulation of genes in the circadian cycle and metabolism[37,39,40]. The RORγT isoform is expressed exclusively in T helper 17 (Th17) cells and regulates expression of interleukins (IL)-17A and IL-17F[41,42] involved in autoimmune disease[43-45]. Pivotal evidence for digoxin involvement in the regulation of RORγT

Table 1 Summary of the main findings from key original articles investigating non-cardiac applications of digoxin and other cardiac glycosides

Manuscript title	Publication year	Main biomedical/molecular findings	Main histological findings	Ref.
Cardiac glycosides inhibit p53 synthesis by a mechanism relieved by Src or MAPK inhibition	2009	Activation of Src/MAPK signaling pathways, resulting in reduction of p53 protein synthesis	NA	[72]
Human cytomegalovirus inhibition by cardiac glycosides: Evidence for involvement of the *HERG* gene	2012	CG reduced expression of the potassium channel gene, *hERG*, and reduced NF-κB levels	NA	[85]
Digoxin Suppresses HIV-1 Replication by Altering Viral RNA Processing	2013	Reduction in HIV-1 viral mRNAs encoding structural proteins, with reduced synthesis of HIV-1 structural protein; altered viral RNA splice site use leading to loss of essential viral factor Rev; changed activity of CLK family of SR protein kinases and modification of SR proteins	NA	[91]
A novel cell-based high-throughput screen for inhibitors of *HIV-1* gene expression and budding identifies the cardiac glycosides	2014	Na-K ATPase- dependent but intracellular Ca2+-independent inhibition of *HIV-1* gene expression at the post-integration stage of the viral life cycle	NA	[90]
Digoxin Suppresses Tumor Malignancy through Inhibiting Multiple Src-Related Signaling Pathways in Non-Small Cell Lung Cancer	2015	Inhibition of proliferation, invasion, migration, and colony formation of A549 lung cancer cells; suppression of Src and related protein activity; reduced EGFR and STAT3 activity	NA	[75]
Synergistic effects of ion transporter and MAP kinase pathway inhibitors in melanoma	2016	Inhibition of the ATP1A1 Na^+/K^+ pump, which is highly expressed in melanoma, resulting in selective toxicity to melanoma cells. Digoxin was also additive or synergistic with MEK inhibitor and/or BRAF inhibitor to induce cell death in melanoma cells; increased intracellular acidification, mitochondrial calcium dysregulation, and ATP depletion in melanoma cells	NA	[79]
Small-molecule TFEB pathway agonists that ameliorate metabolic syndrome in mice and extend *C. elegans* lifespan	2017	Activated TFEB, conferred hepatoprotection against diet-induced steatosis in mice, and extended lifespan of *Caenorhabditis elegans*	Amelioration of high-fat diet-induced steatosis, reversal of hepatocyte p62/SQSTM1 accumulation, suggesting enhanced autophagic flux	[62]
Targeting Intracellular Ion Homeostasis for the Control of Respiratory Syncytial Virus	2018	Findings suggested digoxin-mediated inhibition of RSV transcription and/or replication, likely dependent on changes in intracellular Na+ and K+	NA	[82]
Digoxin Suppresses PKM2 Promoted HIF-1α Transactivation in Steatohep-atitis	2018	Binding of PKM2 by digoxin downregulated HIF-1α transactivation to decrease sterile inflammation in the liver. Digoxin suppressed ROS production both *in vivo* and *in vitro* from hepatocytes and immune cells	Reduction in hepatic damage, steatosis, and inflammation induced by endotoxin, high fat diet, or alcohol	[27]
Digoxin improves steatohep-atitis with differential involvement of liver cell subsets in mice through inhibition of PKM2 transactivation	2019	Digoxin downregulated PKM2-PKM2-HIF-1α axis and attenuated inflammasome activity in macrophages and hepatic oxidative stress response	Reduction of high fat diet-induced hepatic damage, steatosis, and liver inflam-mation	[28]
Antiviral activity of digoxin and ouabain against SARS-CoV-2 infection and its implication for COVID-19	2020	Inhibition of viral mRNA expression, copy number, and viral protein expression at the post entry stage of the viral life cycle	NA	[81]
Classical Drug Digitoxin Inhibits Influenza Cytokine Storm, With Implications for COVID-19 therapy	2020	Suppression of levels of the cytokines TNF-α, GRO/KC, MIP2, MCP1, and IFN-γ during cytokine storm	No difference in density of immune cells in rat lung sections, comparing digitoxin-treated and control lungs	[84]
Inhibition of the IL-17A axis in adipocytes suppresses diet-induced obesity and metabolic	2021	Digoxin inhibition of RORγT activity suppressed the IL-17A axis, thus preventing diet-induced obesity, metabolic alterations, and liver injury	Prevention of high fat diet-induced hepatic lipid accumulation, reduced	[59]

disorders in mice	fibrosis, increased browning of adipose tissue

TFEB: Transcription factor EB; PKM2: Pyruvate kinase M2; IL-17A: Interleukin-17A; MAPK: Mitogen-activated protein kinase; CG: Cardiac glycoside; NF-κB: Nuclear factor-kappaB; CLK: Cdc2-like kinases; SR: Serine-arginine; EGFR: Epidermal growth factor receptor; MEK: MAP kinase kinase; RSV: Respiratory syncytial virus; HIF-1α: Hypoxia-inducible factor 1-alpha; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; TNF-α: Tumor necrosis factor-alpha; GRO/KC: Growth-regulated oncogene/keratinocyte chemoattractant; MIP2: Macrophage inflammatory protein 2; MCP1: Monocyte chemoattractant protein-1; IFNγ: Interferon-gamma; RORγT: Retinoid-related orphan receptor-gamma; NA: No application; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3.



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Figure 1 Digoxin reduces steatohepatitis by suppressing pyruvate kinase M2 dependent hypoxia-inducible factor 1-alpha activity and inhibiting reactive oxygen species production[27,28]. Digoxin structure derived from MolView. HIF-1α: Hypoxia-inducible factor 1-alpha; PKM2: Pyruvate kinase M2; ROS: Reactive oxygen species.

activity was provided by Huh *et al*[46] in 2011 when they showed that digoxin inhibits the transcriptional activity of RORγT[46]. Inhibition of RORγT by digoxin or its non-toxic derivatives selectively inhibits Th17 differentiation, delaying the onset and severity of autoimmune reactions in murine models [46]. More recently, Karaś *et al*[47,48] reported opposing findings with CGs activating RORγ in HepG2 cells and RORγT in Th17 lymphocytes[47,48] when these compounds were used at much lower doses than originally used by Huh *et al*[46]. Thus, it appears that digoxin-mediated inhibition *vs* activation of RORγT may be dependent of the dose utilized[49].

RORγ directly regulates glucose-6 phosphatase (G6Pase) and a number of genes involved in glucose regulation and insulin sensitivity. G6Pase facilitates glucose-6 phosphate hydrolysis into inorganic phosphate and free glucose[50-52], with suppression of hepatic G6Pase resulting in accumulation of glucose-6 phosphate and metabolic reprogramming involving increased carbohydrate response element binding protein activity and gene expression that lead to hepatic steatosis[53-56]. Digoxin-mediated activation of RORγ upregulates G6Pase, resulting in improved glucose homeostasis and decreased NAFLD phenotype.

In many respects, the pathophysiological changes seen in alcohol-associated steatohepatitis (ASH) are similar to those seen in NASH, including increased oxidative stress and sterile inflammation manifested as steatohepatitis[57]. The ability of digoxin to improve ASH was tested in a well-accepted Lieber-Decarli ethanol liquid diet (5% ethanol) plus a single ethanol binge mouse model during chronic feeding [58]. Digoxin (0.2-1.0 mg/kg) dose-dependently improved hepatic steatosis, neutrophil infiltration, and hepatocellular damage in ASH. The effect of digoxin was confirmed in human liver tissues, which showed a greater degree of upregulation of HIF-1α and HIF-1α-dependent genes in severe ASH compared to mild disease. It was concluded that long-term treatment with digoxin reduced chronic liver damage, inflammation, and steatosis in experimental models of NASH and ASH without affecting cardiac chronotropic and inotropy.

Digoxin is notable for producing cardiotoxicity at concentrations that are close to its effective concentration. Remarkably, however, digoxin did not have any cardiac or other toxicity at lower doses. These studies identified an entirely novel application of this old drug at doses significantly below the dose required for the cardiac effect. Digoxin showed the potential to therapeutically inhibit liver injury in both ASH and NASH through the regulation of PKM2-HIF-1α pathway activation with the involvement of multiple cell types. Because of the large clinical experience with oral digoxin, this may have significant clinical applicability in human ASH and NASH. Digoxin is currently being investigated in a phase II pilot study in patients with ASH (NCT05014087) (Table 2).

DIGOXIN IN OBESITY AND METABOLIC DISORDERS

Overnutrition, inadequate physical activity, genetic and epigenetic factors, and other risk factors can

Table 2 Ongoing clinical trials of digoxin in non-cardiac diseases

Study title	Medication doses	Current status	Estimated study completion date	Clinical Trials.gov number	Number of participants	Country of trial
Phase 1						
Inhibition of Sterile Inflammation by Digoxin	Digoxin 3.00 mcg/Kg/day *vs* Digoxin 0.15 mcg/Kg/day *vs* placebo	Recruiting	July 2023	NCT03559868	45	United States
Phase IB Trial of Metformin, Digoxin, Simvastatin in Subjects With Advanced Pancreatic Cancer and Other Advanced Solid Tumors	Metformin 850 mg po/day, Simvastatin 5 mg po/day, Digoxin 0.0625 mg po/day *vs* Metformin 850 mg po/day then 1700 mg po/day, Simvastatin 20 mg po/day, Digoxin 0.25 mg po/day *vs* Metformin 850 mg po/day then 1700 mg po/day Simvastatin 40 mg po/day, Digoxin 0.25 mg po/day then 0.375 mg po/day	Recruiting	December 2023	NCT03889795	15	United States
Effect of Digoxin on Clusters of Circulating Tumor Cells in Breast Cancer Patients	Digoxin 0.125 mg or 0.250 mg digoxin based on renal function and target serum digoxin concentration	Recruiting	June 2022	NCT03928210	9	Switzerland
Phase 2						
Digoxin In Treatment of Alcohol Associated Hepatitis	Digoxin titration to goal 0.5 and 1.1 ng/mL *vs* no digoxin	Recruiting	August 2024	NCT05014087	60	United States
Evaluating the Effect of Digoxin and Ursodeoxycholic Acid in Patients With Rheumatoid Arthritis	Digoxin 0.25 mg + DMARDS *vs* UCDA 500 mg + DMARDS *vs* placebo + DMARDS	Recruiting	July 2022	NCT04834557	90	Egypt
FOLFIRINOX With Digoxin in Patients With Resectable Pancreatic Cancer	FOLFIRINOX + digoxin 0.125 or 0.250 mg for target digoxin level 0.8 to 1.2 ng/mL	Recruiting	February 2025	NCT04141995	20	United States
Topical Ionic Contra-Viral Therapy in Actinic Keratosis	Digoxin topical gel 0.125% *vs* furosemide topical gel 0.125% *vs* digoxin and furosemide gel 0.125% *vs* vehicle gel	Unknown	September 2019	NCT03684772	32	Netherlands
Phase II Multicentric Study of Digoxin Per os in Classic or Endemic Kaposi's Sarcoma (KADIG 01)	Digoxin goal 0.6 to 1.2 ng/mL for age < 75 yr; Digoxin goal 0.5-0.8 ng/mL for age > 75 yr	Unknown	September 2019	NCT02212639	17	France

DMARDS: Disease-modifying antirheumatic drug; FOLFIRINOX: FOLinic acid, 5-Fluorouracil, IRINotecan and Oxaliplatin; UCDA: Ursodeoxycholic acid.

predispose individuals to metabolic syndrome[59] with associated comorbidities[60]. Inhibition of RORγT-mediated IL-17A production by digoxin abolishes the IL-17A axis[46], suppressing diet-induced obesity and leading to increased brown adipose tissue[61]. Brown adipose tissue is an essential site for thermogenesis and critical for maintaining body temperature regulated by mitochondria uncoupling protein-1[61]. The metabolic effects observed with digoxin can also be achieved by the ubiquitous deletion of IL-17 receptor A. Modulation of IL-17A signaling may thus serve as a strategy to inhibit obesity and related complications[59].

Metabolic disorders, including obesity, liver steatosis, and aging, may be improved by caloric restriction or starvation, which activates the transcription factor EB (TFEB) that regulates lipid metabolism and the biogenesis of lysosomes. Agents that activate TFEB can confer metabolic changes resembling starvation and thus have utility in the treatment of these metabolic disorders. Recently through a nanotechnology-enabled high-throughput screening of various small molecules, digoxin was one of three small molecules identified that activate TFEB[62]. This activation occurs through distinct calcium-dependent mechanisms and by promoting autophagolysosomal activity, an adaptive catabolic process that generates nutrients and energy during starvation[62]. Calcium is stored in cells in three different compartments, including lysosomes, mitochondria, and the endoplasmic reticulum[63], and TFEB activators can differentially affect calcium stores in these compartments. Digoxin induces lysosomal calcium release through mucolipin 1, leading to activation of TFEB with resultant anti-obesity effects[59].

CGs also appear to hold promise for heritable metabolic disorders. Familial hypercholesterolemia, characterized by elevated serum low-density lipoprotein-cholesterol, is a genetic disorder caused primarily by mutations in the low-density lipoprotein receptor. Patients with compound heterozygous

or homozygous mutations in the low-density lipoprotein receptor have low-density lipoprotein-cholesterol levels > 500 mg/dL, leading to the formation of xanthomas, severe cardiovascular disease, and early death[64]. Hepatocyte-like cells derived from induced pluripotent stem cells from patients with homozygous familial hypercholesterolemia have been used to screen for potential pharmacological therapies[65]. CGs reduced apoB, the crucial protein component of very-low-density lipoprotein and low-density lipoprotein particles, in human hepatocytes as well as in the serum of mice with humanized livers. The mechanism through which CG-mediated reduction of apoB and improvement of hypercholesterolemia occurred did not appear to involve the expression of the *APOB* gene or the synthesis of apoB protein but rather the enhancement of proteolytic turnover of the apoB protein[65].

DIGOXIN IN AUTOIMMUNE AND INFLAMMATORY CONDITIONS

Th17 cells are an independent subset of T helper cells that produce IL-17 and are involved in the induction of inflammation and autoimmune disease. These cells have a unique transcription factor, RORγT[41], and are activated by IL-6 and transforming growth factor-beta 1. Because Th17 cells are inducers of inflammation and autoimmune disease, specific targeting of these cells can reduce inflammation. Digoxin downregulates Th17 differentiation through suppression of RORγT transcriptional activity without effect on the differentiation of T cell lineages[66].

Th17 and T1 play a crucial role in rheumatoid arthritis, a systemic autoimmune inflammatory disorder characterized by hyperplasia of the synovial membrane along with persistent inflammation of joints. In one study assessing the effect of digoxin on the peripheral blood mononuclear cells of 30 rheumatoid arthritis patients and 10 healthy controls, there was a significant reduction in the population of Th17 cells through suppression of the transcription factor RORγT and a decrease in the levels of IL-1β , IL-6, IL-17, and IL-23 cytokines[67]. Digoxin treatment did not modify the expression of transforming growth factor-beta 1 and interferon-gamma (IFN-γ) at the level of mRNA and protein.

Psoriasis is another chronic inflammatory disease involving IL-17-producing Th17 cells[68]. The toll-like receptor 7 agonist imiquimod creates psoriasis-like lesions on the ear or back skin of mice through an IL-17-dependent mechanism. Intraperitoneal digoxin differentially affects these skin lesions, reducing those on the ear and exacerbating those on the back[68]. This differential effect of digoxin may relate to differences in target tissues, the imiquimod application dose, and digoxin bioavailability in different sites.

Digoxin might also be effective for managing pain[69]. Digoxin is a potent inhibitor of soluble epoxide hydrolase enzyme, which breaks down endogenous lipid mediators like epoxyeicosatrienoic acids that are known to have cardiovascular effects including vasodilation, anti-migratory actions on vascular smooth muscle cells, and anti-inflammatory actions[70]. Digoxin has antipyretic activity in rats and inhibits neutrophil infiltration and alveolar septal thickening in lung tissue[69]. Administration of digoxin at a low dose can reduce pain and allodynia and decrease edema and abdominal contraction [69].

DIGOXIN IN CANCER

In a study investigating potential new drugs for prostate cancer, digoxin was found to be highly potent in inhibiting prostate cancer cell growth *in vitro*[71]. Regular digoxin use, especially over 10 years, was found to be associated with a 25% lower risk of prostate cancer[71]. Although the methods through which digoxin reduced prostate cancer risk are unclear, one potential mechanism involves the increased influx of intracellular calcium into prostate cancer cells triggering apoptosis through the cyclin-dependent kinase 5/p25 pathway. Activated Src/mitogen-activated protein kinase (MAPK) signaling results in inhibition of p53 synthesis, suggesting that CGs may have utility in the treatment of cancers with gain of function *P53* mutations[72]. Other mechanisms proposed for the anticancer effects of digoxin include inhibition of Na^+/K^+-ATPase and topoisomerase[73], alterations of calcium signaling [74], and inhibition of HIF-1α synthesis[26]. The DIG-HIF-1 pharmacodynamic trial, which sought to test whether digoxin can reduce the expression of HIF-1α protein in surgically resected breast cancer tissue, was terminated early due to difficulty with accrual (NCT01763931). We hope that there will be subsequent studies that will shed light on this important question.

When given together with the anti-neoplastic drug adriamycin, digoxin enhanced anti-cancer effects *in vitro* on non-small cell lung cancer by inhibiting both DNA double-strand break and single-strand break repair and reducing the cardiotoxicity of adriamycin[72]. Cotreatment with digoxin blocked the adriamycin-induced reduction in cardiomyocyte size, suggesting that digoxin can ameliorate the reduction of heart weight/body weight ratio by adriamycin.

Digoxin suppresses lung cancer progression by inhibiting Src activation and related pathways[75]. In digoxin-treated cells, the phosphorylation of Src and its related proteins was inhibited, suppressing lung cancer cell proliferation, migration, and invasion through inhibition of phosphatidylinositol 3-kinase, focal adhesion kinase, stress-activated protein kinases/Jun amino-terminal kinases, paxillin, and

p130Cas activities. Digoxin also reduces mRNA expression of Src and related protein kinases[75]. Digoxin was also found to have effects on glioblastoma, a highly aggressive and lethal brain tumor, by enhancing apoptosis and reducing the levels of the anti-apoptotic protein through its proteasomal degradation[76].

A screen of 200000 small molecules for inhibitory effect against primary human melanoma cells showed that several CGs, including digoxin, demonstrated toxicity against melanoma cells *vs* normal human melanocytes[77]. This effect involves inhibition of the ATP1A1 Na^+/K^+ pump that is crucial for the maintenance of ion gradients across the plasma membrane for substrate transport. Although CGs alone were insufficient to cause melanoma regression in patient-derived xenografts, they showed synergistic effects with inhibitors of MAPK pathway to mediate regression in both *BRAF* wildtype and *BRAF* mutant melanomas[77]. Polarization of CD4+ T cells into the Th17 subtype in a transgenic mouse model resulted in destruction of advanced B16 murine melanoma through IFN-γ dependent mechanisms[78]. A recent phase 1B clinical trial of digoxin and trametinib, a MAP kinase kinase inhibitor, in patients with *BRAF* wildtype metastatic melanoma who were refractory or intolerant to immune checkpoint blockade showed that 13 out of 20 patients (65%) achieved disease control (NCT28278423)[79]. The results of this early study are encouraging and need to be expanded.

Digoxin is currently being studied in a phase 1B combination drug trial in pancreatic cancer and other advanced solid tumors (NCT03889795) (Table 2). It is also being studied for feasibility and safety when combined with folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin in patients with resectable pancreatic cancer (NCT04141995).

DIGOXIN IN VIRAL INFECTION

Digoxin inhibits coronaviruses and other viruses[80]. It inhibits the cytokine storm generated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and blocks viral cell penetration and infectivity[81]. After single-dose digoxin treatment, SARS-CoV-2 titers were the same as achieved with treatment by remdesivir, with > 99% viral inhibition compared to controls or patients on chloroquine at 48 h post-infection[81]. In other cases, digoxin suppressed viral mRNA expression (99%) more effectively than remdesivir (> 60%) or chloroquine (> 30%)[82]. Host cell entry by Middle East respiratory syndrome and SARS-CoV is inhibited through the silencing or inhibition of the Na, K-ATPase α1-subunit by low doses of CG. This disruption of cell entry occurs at an early stage by interfering with endocytosis through a non-elucidated pathway[80,83]. In the post-entry stage, digoxin significantly inhibits viral mRNA expression, copy number, and viral protein expression at half-maximal inhibitory concentration of 0.043 nM[81].

In rat models infected with influenza virus, administration of digoxin analog digitoxin suppressed cytokine levels, including tumor necrosis factor-alpha, growth-regulated oncogene/keratinocyte chemoattractant, macrophage inflammatory protein 2, monocyte chemoattractant protein-1, and IFN-γ in the rat lung[84]. The inhibition of Na-K-ATPase by CGs decreased intracellular potassium, inhibiting the host cell translational machinery and decreasing influenza virus replication[80].

Digoxin and other CGs also inhibit replication of cytomegalovirus, a herpesvirus pathologic agent of important human diseases, at nanomolar concentrations, with an additive effect when combined with antiviral drugs for cytomegalovirus such as ganciclovir[80]. CGs reduced the levels of viral proteins and cellular nuclear factor-kappaB, with the activity of CGs correlating with the expression of *hERG*, a potassium channel gene[85].

Human papillomaviruses (HPVs) rely on potassium ion influx for replication[86]. Cutaneous warts (including plantar warts or common warts) are typically caused by HPV 1, 2, 27, and 57[87,88], while genital warts are typically caused by HPV 6 and 11. CGs such as digoxin and the loop diuretic furosemide interact with the cell-membrane ion cotransporters Na^+/K^+-ATPase and Na-K-Cl and inhibit potassium flux thus inhibiting HPV replication[86]. The inhibitory effect on DNA replication appears most potent when digoxin and furosemide are combined; the term ionic contra-viral therapy (ICVT) describes the topical application of these drugs in combination. A phase 1/2 open-label study of ICVT was safe and efficacious in 12 healthy patients with common warts[89]. A follow-up randomized, double-blind, placebo-controlled phase 2A proof-of-concept study assessed the efficacy, safety, and tolerability of ICVT in adults with cutaneous warts. Eighty adult patients were randomized to digoxin or furosemide alone, ICVT or placebo (NCT02333643)[87]. Reduction in HPV load and wart size was achieved in all active treatment groups but not in placebo, with a statistically significant reduction in wart diameter in those treated with ICVT *vs* placebo. On the contrary, a phase 2 study of ICVT for HPV-related genital lesions was terminated early due to a lack of effect on interim analysis (NCT03334240). Overall, digoxin appears promising for the treatment of HPV-induced lesions, especially the cutaneous subtype, and warrants further investigation in large multicenter studies.

A cell-based screen performed on cells transfected with proviral DNA constructs uncovered a number of compounds that inhibit HIV-1 virion production, including numerous CGs[90]. Digoxin selectively impaired HIV-1 replication at two levels: (1) Through global alterations in the efficiency of HIV-1 RNA processing; and (2) By blocking the export of incompletely spliced viral RNAs to the

cytoplasm[91]. The cardenolides and the bufadienolides, both subclasses of CGs, inhibited the late stages of the HIV-1 replication cycle. Although both are C(23) steroids, they differ in that cardenolides contain a five-membered lactone ring at C-17, whereas bufadienolides contain a six-membered lactone ring. Members of both classes of CGs inhibited late stages of HIV-1 production, and changes in structure resulted in changes in inhibition. Digoxin (and potentially the CG family of drugs) represents a novel HIV-1 inhibitor with the potential for rapid development into antiretroviral therapy. The dose-limiting toxicities observed with CGs in humans are typically related to toxic increases in cardiac contractility driven by increases in intracellular calcium. As the mechanism of CG inhibition of HIV-1 appears to be independent of such calcium increases, it is possible that structural modification of the CGs could avoid cardiac toxicity while maintaining HIV-1 inhibition.

DIGOXIN IN NON-CARDIAC GENETIC DISORDERS

CGs or their derivatives, including digoxin, also appear promising for treating certain genetic diseases, such as cystic fibrosis and Duchenne's muscular dystrophy, wherein truncated protein products encoded by the corresponding nonsense mRNAs are fully or partially functional[92,93]. The nonsense-mediated mRNA decay (NMD) pathway selectively eliminates aberrant transcripts containing premature translation termination codons and regulates the levels of a number of physiological mRNAs. NMD modulates the clinical outcome of a variety of human diseases, including cancer and several genetic disorders. Using a dual-color bioluminescence-based NMD reporter system, Nickless *et al*[94] performed a high-throughput screen to identify drug candidates that can alter NMD activity in human cells[94]. The effects of seven of the inhibitor hits were found, and each validated compound inhibited NMD in a dose-dependent manner. Notably, the top five verified hits, including digitoxin, digoxin, lanatoside C, proscillaridin, and ouabain, are all CGs[95]. It should be noted that the concentrations of CGs used in this study to achieve more complete NMD inhibition without causing significant cellular toxicity (for example, 500 nM for digoxin and 175 nM for ouabain) are much higher than standard clinical doses used for the treatment of cardiac failure. Thus, acute use of these drugs at the experimental working concentrations cannot directly translate to the clinic owing to *in vivo* toxic effects. However, the benefits of partial NMD inhibition with chronic treatment at clinically relevant doses may potentially be efficacious, but this will require further clinical pharmacology studies.

CONCLUSION

Until now, most of our knowledge and experience with digoxin pertains to its use in the cardiac field. However, in the past decade, digoxin has emerged as a potential pharmacologic agent in the management of several conditions, including steatohepatitis in the context of nonalcohol and alcohol-associated fatty liver disease, obesity and other metabolic disorders, autoimmune conditions, malignancy, and viral infection, among others. Clinical trials on the repurposing of digoxin for therapeutic use in a variety of non-cardiac conditions are still in their early stages but appear promising.

At relatively high concentrations (hundreds of nM), digoxin and other CGs inhibit the Na-K ATPase pump, leading to accumulation of sodium ions in the cytosol that drives an influx of calcium into the heart, increasing contractility[96]. At lower doses (picomolar to low nanomolar), digoxin induces the Na-K ATPase to act as a receptor that can modulate a variety of pathways[5,96], including the Src/MAPK pathway, which regulates a number of downstream signaling pathways. Also at high doses, digoxin binding to the ligand-binding domain of the NR RORγT inhibits its transcriptional activity, leading to inhibition of Th17 activity and IL-17 release[59] and suppressing nuclear factor-kappaB activity[85], altogether reducing the inflammatory response. At lower doses, digoxin activates RORγT signaling, leading to induction of several Th17-specific genes, suggesting a potential role of digoxin in adoptive cell therapy[14,47,48].

Several questions remain to be clarified in the quest towards repurposing of digoxin including: the structure-activity relationships that direct its molecular targeting in specific disease settings; whether dosing/concentration alone determines its activity as an inhibitor *vs* activator or whether other factors affect its action; and the ideal potency that can be utilized for pharmacologic intervention in a particular tissue while optimizing its safety profile. Indeed, the decline of digoxin in the cardiac arena is largely attributable to its narrow therapeutic index and potential toxicity, thus it is very exciting that recent studies show potent biological activity of much smaller doses of digoxin than used historically in the clinical setting. Digoxin is commercially available as a relatively cheap generic drug, thus further elucidation of its biological effects and mechanisms of action especially at low non-toxic doses will facilitate its rapid therapeutic repurposing.

FOOTNOTES

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MINIREVIEWS

Autoimmune liver diseases and SARS-CoV-2

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), can trigger autoimmunity in genetically predisposed individuals through hyperstimulation of immune response and molecular mimicry. Here we summarise the current knowledge about autoimmune liver diseases (AILDs) and SARS-CoV-2, focusing on: (1) The risk of SARS-CoV-2 infection and the course of COVID-19 in patients affected by AILDs; (2) the role of SARS-CoV-2 in inducing liver damage and triggering AILDs; and (3) the ability of vaccines against SARS-CoV-2 to induce autoimmune responses in the liver. Data derived from the literature suggest that patients with AILDs do not carry an increased risk of SARS-Cov-2 infection but may develop a more severe course of COVID-19 if on treatment with steroids or thiopurine. Although SARS-CoV-2 infection can lead to the development of several autoimmune diseases, few reports correlate it to the appearance of *de novo* manifestation of immune-mediated liver diseases such as autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) or AIH/PBC overlap syndrome. Different case series of an AIH-like syndrome with a good prognosis after SARS-CoV-2 vaccination have been described. Although the causal link between SARS-CoV-2 vaccines and AIH cannot be definitively established, these reports suggest that this association could be more than coincidental.

Key Words: Autoimmune liver disease; SARS-CoV-2; COVID-19; COVID-19 vaccine; Autoimmune hepatitis

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Core Tip: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a possible trigger of autoimmunity. Patients with autoimmune liver diseases (AILDs) were considered at higher risk of SARS-CoV-2 infection and more susceptible to severe coronavirus disease 2019 (COVID-19) due to genetic background and immunosuppressive treatments. Case reports documenting autoimmune hepatitis-like syndromes after the COVID vaccine started to emerge, raising worries about a possible risk of unwanted immunological side effects, especially in individuals predisposed to autoimmune disorders. We herein discuss the consequences of SARS-CoV-2 infection in patients with AILDs and the role of the vaccines in inducing liver autoimmunity.

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INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded RNA, enveloped, beta coronavirus causing coronavirus disease 2019 (COVID-19), which has developed into a pandemic infection in a short time since its first appearance in Wuhan, China, in December 2019[1,2]. Since then, SARS-CoV-2 has caused more than 620 million infections, with a death toll exceeding 6.5 million as of October 31, 2022, according to the World Health Organization. Although the most common presentation of COVID-19 includes respiratory symptoms such as dry cough and shortness of breath[3], gastrointestinal symptoms, including diarrhoea, anorexia, nausea and or vomiting, and abdominal pain, have been variably reported in up to half of the cases[4,5]. Furthermore, elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hallmarks of liver injury, have been widely recognised as major components of COVID-19[6]. Tissue injury is almost certainly multifactorial and related to several mechanisms, such as direct hepatocellular lesions, immune-mediated liver damage due to the severe inflammatory response, or development of endotheliopathy with hypoxic or ischaemic injury[7-9].

Furthermore, SARS-CoV-2 has also been identified as an instrumental trigger of autoimmunity. Hyper-stimulation of the immune system or molecular mimicry of the virus with human self-components may lead to the synthesis of multiple autoantibodies that could initiate autoimmune diseases in genetically predisposed individuals[10,11].

In this review, we summarised the current knowledge regarding the risk of SARS-CoV-2 infection and the course of COVID-19 in patients with autoimmune liver diseases (AILDs), as well as the role of SARS-CoV-2 in inducing *de novo* AILDs. Finally, since various case reports documenting autoimmune hepatitis (AIH)-like syndromes after receiving the COVID-19 vaccine started to emerge, we reviewed the literature to analyse the role of vaccines in inducing autoimmune responses in the liver.

SARS-COV-2 AND PRE-EXISTING AILDS

ALDs are a heterogeneous group of inflammatory liver disorders mediated by autoimmunity, including AIH, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and overlapping syndromes. Patients with AILDs could theoretically present an increased risk of SARS-CoV-2 infections and poor outcomes due to lifelong immunosuppressive treatments taken to delay the progression to cirrhosis and liver failure.

However, the clinical impact of SARS-CoV-2 infection on the population with AILDs remains unclear. Preliminary data derived from regional observational studies driven by referral centres suggested that patients with AILDs did not carry a specific risk of becoming infected by SARS-CoV-2. In the earliest reports from Northern Italy, an area particularly affected by the virus's spread, the observed incidence of the infection in a cohort of 148 AILDs patients (133 AIH, 11 autoimmune sclerosing cholangitis, 2 PSC/AIH and 2 PBC/AIH) did not significantly differ from that estimated in the general population (43 *vs* 38 cases, respectively)[12]. Similarly, Verhelst *et al*[13] found a very limited SARS-CoV-2 infection ratio (1.2%) in a Belgian cohort of 110 patients with AIH[13]. More recently, a survey involving 1779 AILDs patients from 20 European countries detected a rate of COVID-19 (2.2%) equivalent to the period prevalence of the general population, thus indicating that patients with AILDs are not at elevated risk of developing COVID disease[14]. Interestingly, people suffering from autoimmune diseases do not have a higher risk of being positive for COVID-19, as indicated by a study performed in Milan that applied both a test-negative [odds ratio (OR): 0.86, 95% confidence interval (CI): 0.76–0.96] and population (OR: 0.98, 95%CI: 0.90–1.08) case–control design[15].

Regarding the impact of AILDs on the COVID-19 course, early phone-based surveys including patients with AIH (n = 73) and PBC (n = 68) found a rate of infection of 3.6% with a favourable outcome of COVID-19 in most cases[16]. Likewise, patients under immunosuppressive treatment for AIH show a disease course probably comparable to that reported in the non-immunosuppressed population[17]. Data combined from 3 large-scale international reporting registries, namely the European Association for the Study of the Liver supported COVID-Hep registry, the European Reference Network on Hepatological Diseases (ERN RARE-LIVER) and the American Association for the Study of Liver Diseases supported SECURE-cirrhosis registry, showed that patients with AIH, despite immunosuppressive treatment, did not present an increased risk of adverse outcomes including hospitalisation (76% *vs* 85%; P = 0.06), admission in an intensive care unit (29% *vs* 23%; P = 0.240), or death (23% *vs* 20%; P = 0.643) when compared to other causes of chronic liver disease and matched cases without the liver disease [18]. Another retrospective study collecting data on 110 patients with AIH and COVID-19 from 34 centres in Europe and the Americas confirmed that AIH was not a risk factor for severe COVID-19 (15.5% *vs* 20.2%, P = 0.231) and all-cause mortality (10% *vs* 11.5%, P = 0.852) than other causes of chronic liver disease. Still, continued immunosuppression during COVID-19 was associated with a lower rate of liver injury (P = 0.009; OR: 0.26; 95%CI: 0.09-0.71)[19]. However, in another study including 254 AIH patients, the same author observed that the use of systemic glucocorticoids (adjusted OR: 4.73, 95%CI: 1.12-25.89) and thiopurine (adjusted OR: 4.78, 95%CI: 1.33-23.50) was associated with worse COVID-19 outcome compared to patients who were not on immunosuppressive therapy[20].

These results might aid medical decisions when dealing with SARS-CoV-2 infection in immunocompromised patients. Although current expert recommendations advocate against changes in immunosuppressive treatments in patients with AIH before and after SARS-CoV-2 infection[21], data derived from larger case series support the notion of maintaining remission with the lowest effective glucocorticoid dose in AIH patients during the COVID-19 pandemic.

Nonetheless, it should be considered that cirrhotic patients, irrespective of the cirrhosis aetiology, once infected with SARS-CoV-2, are more vulnerable than the general population or patients without cirrhosis. Indeed, they have a higher risk of decompensation of liver disease and developing a more severe COVID-19 with a mortality rate of up to 30%[22,23].

SARS-COV-2 AS A TRIGGER OF AILDS

Increasing evidence indicates that SARS-CoV-2 may have a trigger effect of, possibly pre-existing, autoimmune disease, including Guillain-Barré syndrome, systemic lupus erythematosus, myasthenia gravis, large vessel vasculitis and thrombosis, Graves' disease, sarcoidosis and inflammatory arthritis [24].

It is well-known that SARS-CoV-2 infection can result in hyperstimulation of the immune system, with increased serum concentration of pro-inflammatory cytokines, particularly monocyte chemoattractant protein 1, interferon-inducible protein-10, interleukin (IL)-6, IL-1β, IL-8, IL-10, IL-17, tumour necrosis factor, granulocyte-macrophage colony-stimulating factor, also referred to as "cytokine storm" or "cytokine release syndrome"[25]. In addition, due to structural homology between some components of SARS-CoV-2 and human proteins[11], the immune responses raised against the virus can cross-react with self-proteins, a concept commonly termed molecular mimicry[26]. These factors may contribute to the development of multiple autoantibodies with a trigger effect on autoimmunity (Figure 1).

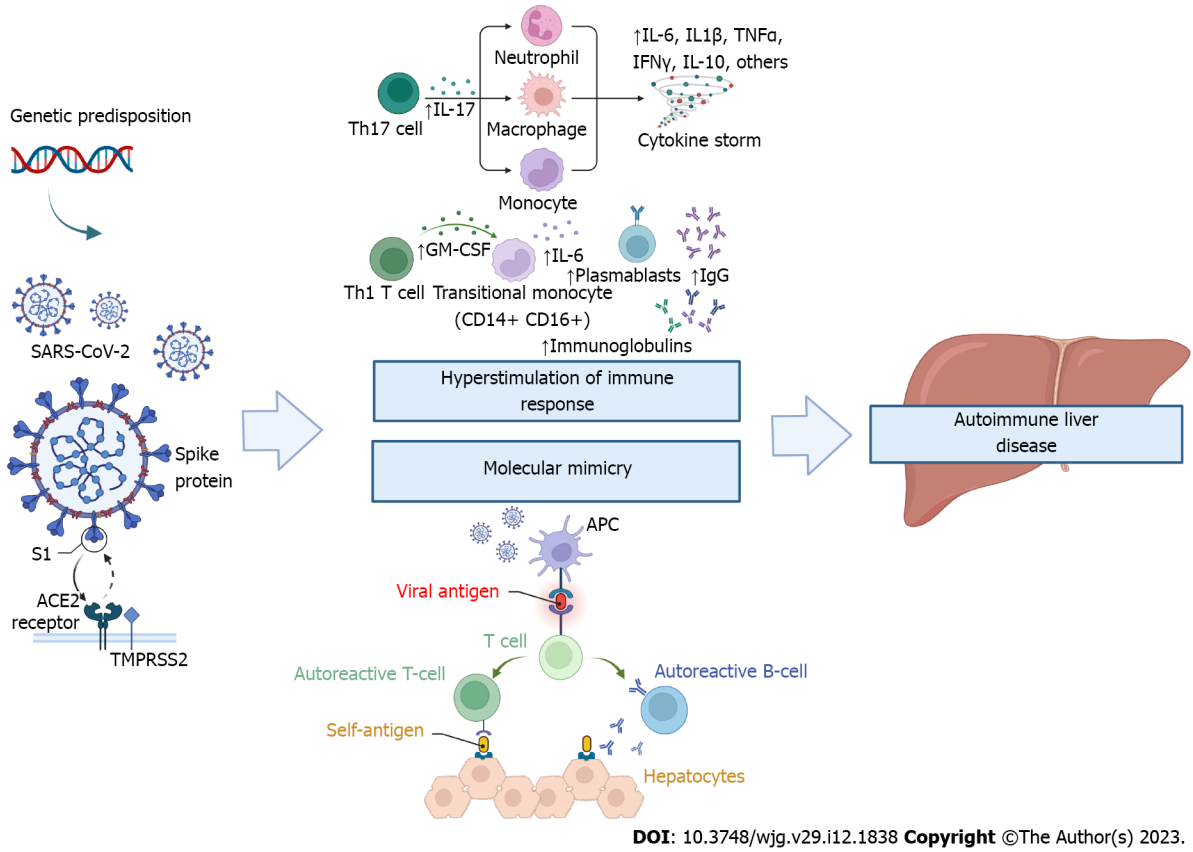
In patients with SARS-CoV-2 infection and especially severely ill patients compared with those with mild or moderate disease, numerous autoantibodies, including antinuclear antibodies (ANA), anti-neutrophil cytoplasmic antibodies, antiphospholipid antibodies, have been detected[27,28]. Since systemic autoimmunity is known to initiate from generalised polyclonal B cell activation, the presence of autoantibodies in such kinds of patients may indicate a pre-AID[29].

However, interestingly, there have been very few reports suggesting that SARS-CoV-2 infection might reflect 'a final hit' leading to de novo manifestation of immune-mediated liver diseases such as PBC[30] or AIH/PBC overlap syndrome[31].

PBC developed concomitantly with Guillain-Barrè syndrome following SARS-CoV-2 infection in a 44-year-old obese and hypertensive woman already suffering from Hashimoto's thyroiditis. During her hospitalisation for severe bilateral interstitial pneumonia, she developed an important rise in serum gamma-glutamyl-transferase (GGT) with initially normal alkaline phosphatase (ALP) levels that progressively increased later.

As a result of the ALP increase combined with high-titre anti-mitochondrial (AMA) positivity (1:640), the patient underwent a liver biopsy showing histological findings consistent with an initial phase (stage I in Scheuer and Ludwig classifications) of PBC.

A case of AIH/PBC overlap syndrome has been described in a 57-year-old man suffering from hypertension, beta-thalassemia minor, and diabetes. He developed arthralgias, elevated liver enzymes and hyper hyperferritinemia one month after recovering from moderate COVID-19 disease. Although a liver biopsy was not performed, the patient presented a concomitant seropositivity for AMA and anti-double-stranded DNA antibodies (anti-ds DNA) that can be considered the serological profile of AIH/



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Figure 1 The severe acute respiratory syndrome coronavirus 2 infection may lead to autoimmune liver disease through hyperstimulation of the immune system and molecular mimicry with human self-components. S1: Spike protein subunit 1; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane Serine Protease 2; TH: T helper cells; IL: Interleukin; TNF-α: Tumour necrosis factor alfa; IFNγ: Interferon-gamma; APC: Antigen-presenting cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IgG: Immunoglobulin G; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

PBC[32].

On the other hand, liver involvement has been frequently reported in COVID-19. Abnormal liver function test, mainly elevated ALT, hypoalbuminemia, and elevated GGT, has been described in 14.8-53% of SARS-CoV-2 infected patients and up to 78% of those with severe manifestations of COVID-19 disease[8] being independent risk factors for adverse clinical outcomes such as intensive care unit admission, use of invasive mechanical ventilation or longer hospital stay[33].

The pathogenic mechanisms of liver injury during COVID-19 are not fully understood. SARS-CoV-2 cell entry is mediated by the interaction of the virus's spike (S) protein with the host angiotensin-converting enzyme 2 receptor (ACE2). S protein is then cleaved by the transmembrane serine protease 2, allowing the internalisation by endocytosis and the release of the viral genome from the endosome[10]. The liver is a possible target for SARS-CoV-2 due to a high expression, almost in the same percentage of type 2 pneumocytes, of ACE2 receptors in cholangiocytes and, to a lesser extent, hepatocytes[34].

However, rather than direct cytotoxicity derived from active viral replication of SARS-CoV-2 in the liver, current data suggest that tissue injury is likely due to immune dysregulation or cytokine storm and development of endotheliopathy and microthromboses with hypoxic or ischaemic injury[35,36]. Exacerbation of underlying liver disease or drug-induced liver injury can also contribute[37,38].

AILDS FOLLOWING SARS-COV-2 VACCINES

Given the dramatic socio-economical effect of the pandemic, vaccines against SARS-CoV-2 have been rapidly developed. In December 2020, two mRNA vaccines (BNT 162b2 Pfizer-BioNTech[39] and mRNA-1273 Moderna[40]) and one adenovirus (ADV) vector-based vaccine (ChAdOx1 nCOV-19 Oxford University/Astra Zeneca)[41] obtained approval under Emergency Use Listing from the most important drug regulatory agencies. As of October 31, 2022, approximately 68% of the global population has received at least one dose of the anti-SARS-CoV-2 vaccine, with 12.9 billion doses being administered worldwide[42].

Both the mRNA and ADV vaccines encode the intracellular production of the SARS-CoV-2 spike protein, the primary target for neutralising antibodies generated from natural infection and triggering both innate and adaptive responses. *Via* their recognition by innate intracellular sensors, including toll-like receptors 3, 7, 9 and inflammasome components, vaccines stimulate innate immunity through cellular activation and consequent release of interferon I or other pro-inflammatory cytokines, thus promoting differentiation of CD4+ and CD8+ T cells into effector and memory subsets[43].

Although the safety profiles of all vaccines have been extensively studied in large placebo-controlled trials, after their widespread diffusion, case reports and case series describing a range of liver diseases following COVID-19 vaccination started to emerge[44].

In the summer of 2021, Bril *et al*[45] first described an AIH-like syndrome developed after vaccination against SARS-CoV-2[45]. AIH is an inflammatory disease of the liver characterised by circulating autoantibodies, mainly ANA, elevated serum globulin levels, specific histological alterations and response to immunosuppressive therapy. The clinical picture is non-specific and varies among patients, ranging from asymptomatic liver enzyme elevation to acute liver failure[46]. SARS-CoV-2 vaccination has also been linked to other forms of immune-mediated liver injury, such as AIH-PBC overlap syndrome[47] or a specific CD8+ T-cell-dominant hepatitis[48].

Cases of *de novo* AIH or vaccine-induced AIH relapse after vaccination against SARS-CoV-2 are reported in Table 1[45,49-78]. A summary of patient information and disease presentation is shown in Table 2. Laboratory data, treatment, and outcome are reported in Table 3.

Most patients (83%) were female, with a mean age of 58.7 years (range 27-82). In 25 patients, race and ethnicity were not specified, whereas, among the remaining cases, 11 patients were Caucasian, three were Asian, and 1 was Arabic. Seventeen patients (42.5%) had a history of either liver (6) or autoimmune disease (11). One patient was three months postpartum[45].

Although five patients were taking potentially hepatotoxic medication, including substitutive hormonal therapy[62], statin[49,58,76,77], azathioprine[51,76], pegylated interferon for polycythaemia vera[53] and nitrofurantoin[73], each patient had been taking their medications for a long time without recent regimens changes. The patient who reported nitrofurantoin completed a three-day course nearly 90 d before the vaccine, and thus nitrofurantoin-induced liver injury was considered highly improbable. Two patients[63,73] took acetaminophen soon after receiving the first dose, whereas one patient[78] was on chronic treatment. The trigger vaccine was Pfizer-BioNTech in 17 cases (43%), Moderna in 11 (28%), Oxford AstraZeneca in 10 (20%), while CoronaVac, an inactivated whole-virion vaccine, was the trigger of AIH in two cases (5%).

Symptomatic AIH was observed in most cases (78%), with a latency time after receiving the COVID-19 vaccine of about two weeks (16.6 ± 12.8 d, range 2-60). The most common symptoms were jaundice (60%), asthenia/fatigue (33%), choluria (25%) and pruritus (20%), whereas other symptoms such as fever, abdominal pain, nausea, and vomiting were variably reported. Interestingly, two patients presented worsening symptoms after receiving the second dose[62,68]. At the laboratory, most cases showed a hepatocellular pattern of liver injury, with markedly elevated transaminases (mean values of AST and ALT of 936 ± 521 U/L and 1060 ± 567 U/L, respectively). Mean GGT, ALP, and bilirubin were 312 ± 220 U/L, 209 ± 95 U/L, and 9.8 ± 8.5 mg/dL, respectively. Immunoglobulin G was > 20 g/L in 13 patients (46.5%) with a mean value of 21.7 g/L. Thirty-seven patients (93%) had at least one positive autoantibody, and ANA was positive in 33 (83%). The scoring systems, such as the Simplified AIH score [79] and the Revised Original Score[80], guided the diagnosis of AIH in 18 cases. A liver biopsy was performed in almost all cases (98%). Findings were typical with AIH in 3 patients and compatible with AIH in 31 cases.

Steroids were used as a first-line treatment in 38 patients (95%), and azathioprine was used as a second agent in 8 patients. The mean time to the first improvement and disease resolution were 4.6 and 45.9 d, respectively. Improvement in laboratory data or complete resolution was seen in 37 out of 40 patients (93%). Three patients died of AIH triggered by viral vector vaccine (AstraZeneca), one after refusing liver transplantation[66], while the other two died of liver failure and sepsis[52,76].

Notably, vaccine-induced AIH has been previously described following other vaccinations than that against SARS-CoV-2, such as hepatitis A[81,82] or influenza virus[83,84].

Although the precise mechanisms have not been elucidated, molecular mimicry is one of the major explanations of autoimmunity after vaccination. Significant homology of amino acid sequences between determinants of vaccines and self-antigen may result in the synthesis of anti-spike antibodies that cross-react with structurally similar host peptide proteins[85]. Other proposed mechanisms include bystander activation (defined as an intense stimulation of innate immunity after administration of adjuvants) and epitope spreading (immune responses to endogenous epitopes resulting from the release of self-antigens during chronic autoimmune/inflammatory processes)[86].

Cases of AIH reactivation after vaccines underline the necessity for close follow-up in individuals with a pre-existing diagnosis of AILDs.

Current knowledge does not allow us to clearly define whether these cases represent a true AIH triggered by vaccine or transient vaccine-induced liver injury, which could have similar clinical, laboratory and histological characteristics.

Table 1 Cases of autoimmune hepatitis-like syndromes after the coronavirus disease 2019 vaccine

Ref.	Sex, age	Comorbidity	Vaccine	Time onset after vaccination	Symptoms	Antibodies	Histology	Treatment	Outcome
Bril *et al*[45]	F, 35	None	Pfizer-BioNTech	7 d after dose I	Jaundice, pruritus, choluria	ANA, anti-Ds-DNA	Compatible with AIH	Prednisone	Remission
Ghielmetti *et al*[49]	M, 63	Type II diabetes, ischemic heart disease	Moderna	7 d after dose I	Jaundice, fatigue, anorexia	ANA	Typical for AIH	Prednisone	Remission
Avci and Abasiyanik [50]	F, 61	Hashimoto thyroiditis, hypertension	Pfizer-BioNTech	30 d after dose I	Jaundice, malaise, fatigue,anorexia	ANA, ASMA	Compatible with AIH	Prednisone + AZA	Remission
Mahalingham *et al*[51]	F, 32	OLT post-AIH, hypertension	Pfizer-BioNTech	21 d after dose III	/	SLA/LP	Compatible with AIH	Methylprednisolone	Remission
Erard *et al*[52]	F, 80		Pfizer-BioNTech	10 d after dose II	All asthenia, pruritus, jaundice	ANA	Compatible with AIH	Steroids	Remission
	F, 73		Moderna	21 d after dose I		ANA		Steroids	Remission
	F, 68		AstraZeneca	20 d after dose I		ANA, ASMA		Listing for liver transplantation	Death after 3 d
Garrido *et al* [53]	F, 65	Polycythaemia Vera	Moderna	14 d after dose I	Abdominal pain, jaundice, pruritus, acholia, choluria	ANA	Compatible with AIH	Prednisolone	Remission
Goulas *et al* [54]	F, 52	/	Moderna	14 d after dose I	Malaise, jaundice	ANA, ASMA		Prednisolone + AZA	Remission
Cao *et al*[55]	F, 57	AIH	CoronaVac	14 d after dose I	Choluria, acholia, jaundice	ANA	Compatiblewith AIH	Methylprednisolone + AZA + UDCA	Remission
Vuille-Lessard *et al* [56]	F, 76	Hashimoto thyroiditis, urothelial ca.	Moderna	3 d after dose I	Choluria, fatigue, jaundice	ANA, ASMA, ANCA	Typical for AIH	Prednisolone + AZA	Remission
Suzuki *et al* [57]	F, 80	GERD	Pfizer-BioNTech	10 d after dose II	Jaundice	ANA	Compatible with AIH	Prednisone	Remission
	F, 75	Dyslipidemia	Pfizer-BioNTech	4 d after dose II	Choluria	ANA, AMA		Prednisone	Remission
	F, 78	Primary biliary cholangitis	Pfizer-BioNTech	7 d after dose I	Fever, malaise	ANA, AMA		Prednisone	Remission
Tan *et al*[58]	F, 56	/	Moderna	42 d after dose I	Jaundice, malaise, anorexia	ANA, AMA	Compatible with AIH	Budenoside	Remission
Zhou *et al*[59]	F, 36	Primary sclerosing cholangitis, ulcerative colitis	Moderna	11 d after dose I	/	ANA, Ds-DNA	Compatible with AIH	Prednisone + Azathioprine	Remission
Kang *et al*[60]	F, 27	/	Pfizer-BioNTech	8 d after dose II	Nausea, vomiting, headache, fever, dark urine	ANA	Compatible with AIH	Prednisolone	Remission
Rocco *et al* [61]	F, 80	Hashimoto thyroiditis Glomer-ulonephritis	Pfizer-BioNTech	7 d after dose II	Jaundice	ANA	Typical for AIH	Prednisone	Remission
Londoño *et al* [62]	F, 41	Premature ovarian failure	Moderna	7 d after dose II	Choluria, jaundice, epigastric pain, nausea, and vomiting	ANA, AMA, SLA/LP	Typical for AIH	Prednisone	Remission
McShane *et al*	F, 71	/	Moderna	4 d after	Jaundice	ASMA	Compatible	Prednisolone	Remission

[63]				dose I			with AIH		
Clayton-Chubb *et al* [64]	M, 36	Hypertension	AstraZeneca	26 d after dose I	Fever	ANA	Compatible with AIH	Prednisolone	Remission
Palla *et al*[65]	F, 40	Sarcoidosis	Pfizer-BioNTech	30 d after dose II	/	ANA	Compatible with AIH	Prednisolone	Remission
Rela *et al*[66]	F, 38	Hypothyroidism	AstraZeneca	20 d after dose I	Jaundice, fever, fatigue	ANA	Compatible with AIH	Prednisolone	Remission
	M, 62	Diabetes	AstraZeneca	16 d after dose I	Anorexia, fever, jaundice	/		Prednisolone	Death after 3 wk
Camacho-Domínguez *et al*[67]	M, 79	/	AstraZeneca	15 d after dose I	Abdominal pain, pruritus, acholia, choluria	ANA, ASMA	Compatible with AIH	Hydrocortisone; Prednisone; Prednisolone	Remission
Zin Tun *et al* [68]	M, 47	/	Moderna	3 d after dose I and 3 d after dose II	Malaise, jaundice	ANA	Compatible with AIH	Prednisolone	Remission
Torrente *et al* [69]	F, 46	Hypothyroidism	AstraZeneca	21 d after dose I	/	ANA	Compatible with AIH	Prednisone + AZA	Remission
Fimiano *et al* [70]	F, 63	Hypothyroidism	Pfizer-BioNTech	54 d after dose III	Abdominal pain, nausea, jaundice, choluria	-	Compatible with AIH	Methylprednisolone + AZA	Remission
Izagirre *et al* [71]	M, 72	Ischemic heart disease, 30 gr/day alcohol consumption	Pfizer-BioNTech	46 d after dose II	/	ANA	Compatible with AIH	Prednisone+ Azathioprine	Remission
	F, 62	Celiac disease	AstraZeneca	4 d after dose II	/	ANA		Prednisone+ AZA	Remission
	F, 72	/	Pfizer-BioNTech	14 d after dose II	/	ANA		Prednisone	Remission
	F, 59	Hypothyroidism	Pfizer-BioNTech	9 d after dose I	/	ANA	Not performed	No treatment received	Spontaneous improvement
Hasegawa *et al*[72]	F, 82	Hepatitis C virus	Pfizer-BioNTech	7 d after dose I	Malaise	ANA	Compatible with AIH	Prednisone	Remission
Shroff *et al* [73]	F, 59	/	Moderna	31 d after dose I	/	ANA	Compatible with AIH	Steroids	Remission
Efe *et al*[74]	M, 53	/	Pfizer-BioNTech	30 d after dose II	Abdominal pain, pruritus, erythematous skin eruption	/	Compatible with AIH	Prednisolone	Developed HE and liver transplantation
Shahrani *et al* [75]	F,59	Dyslipidemia	AstraZeneca	12 d after dose II	Jaundice	/	Compatible with AIH	Prednisolone	Remission
	F,72	/	Pfizer-BioNtech	10 d after booster	Jaundice	AMA	Compatible with AIH	Prednisolone	Remission
Nik *et al*[76]	F,63	Primary sclerosing cholangitis, ulcerative colitis	AstraZeneca	14 d after dose I	Jaundice, pruritus	ANA	Compatible with AIH	Prednisolone	Death after 2 wk for sepsis
Mekritthikrai *et al*[77]	F,52	Dyslipidemia, hypertension	Coronavac	After dose II	Jaundice	ANA, ASMA	Compatible with AIH	Prednisolone + AZA	Remission
Mathew *et al* [78]	F, late 20s	Headache	AstraZeneca	10 d after dose I	Jaundice	ANA	Compatible with AIH	Prednisolone	Remission

ANA: Antinuclear antibody; anti-Ds-DNA: Anti-double stranded DNA; AIH: Autoimmune hepatitis; ASMA: Anti-smooth muscle antibody; AZA: Azathioprine; OLT: Orthotopic liver transplantation; SLA/LP: Anti-soluble liver antigen/liver-pancreas; UDCA: Ursodeoxycholic acid; ANCA: Anti-neutrophil cytoplasmic antibodies; GERD: Gastroesophageal reflux disease; AMA: Anti-mitochondrial antibodies; HE: Hepatic encephalopathy.

Table 2 Cases of autoimmune hepatitis-like syndromes after coronavirus disease 2019 vaccine: Demographics, patient information, and disease presentation

Classification	
Number of patients	40
Females, *n* (%)	32 (83)
Age (years, mean ± SD)	58.7 ± 16
Race/ethnicity, *n* (%)	
Caucasian	11 (27)
Asian	3 (8)
Arabic	1 (3)
Unspecified	25 (62)
Predisposing conditions, *n* (%)	
History of liver disease	6 (15)
History of autoimmune disease	11 (28)
Medications, *n* (%)	
Acetaminophen	3 (8)
Other hepatotoxic drugs (statins, peg-IFN, AZT)	5 (13)
Vaccine, *n* (%)	
Pfizer-BioNTech	17 (43)
Moderna	11 (28)
AstraZeneca	10 (25)
Coronavac	2 (5)
Symptom onset (days after a dose, mean ± SD)	16.6 ± 12.8
Symptoms, *n* (%)	
Jaundice	24 (60)
Astenia/Malaise/Fatigue	13 (33)
Choluria	10 (25)
Pruritus	8 (20)
Abdominal pain	4 (10)
Fever	5 (13)
Loss of appetite	4 (10)
Nausea/vomiting	5 (13)

Peg-IFN: Pegylated interferon; AZT: Azathioprine.

A longer follow-up could be helpful to rule out vaccine-induced liver injury since biochemical and histological resolution can spontaneously occur, and immunosuppressive treatment can be withdrawn without the risk of relapse.

In summary, although a causative link between AIH and SARS-CoV-2 vaccination cannot be confirmed, emerging case reports suggest this association could be more than coincidental.

CONCLUSION

Since the initial stage of the COVID-19 pandemic, concerns about the risk of poorer outcomes following SARS-CoV-2 infection have been raised in patients with pre-existing chronic liver disease. We found that people with AILDs are not at increased risk of being infected by SARS-CoV-2, despite immunosuppressive treatment that, however, may predispose them to develop a more severe course of the disease.

Table 3 Cases of autoimmune hepatitis-like syndromes after coronavirus disease 2019 vaccine: Diagnostics, treatment, and clinical outcomes

Diagnostics	
Peak laboratory values (mean ± SD)	
AST (IU/l)	936 ± 523
ALT (IU/l)	1060 ± 567
GGT (IU/l)	312 ± 220
ALP (IU/l)	209 ± 95
Bilirubin (mg/dL)	9.8 ± 8.5
IgG (g/L)	21.7 ± 6.7
Antibodies, *n* (%)	
ANA	33 (83)
ASMA	7 (18)
SLA/LP	2 (5)
AMA	5 (13)
Others (ANCA, DS-DNA)	3 (8)
Liver biopsy	
Performed	39
Not performed	1
Morphologic characteristics, *n* (%)	
Interface hepatitis	31 (78)
Lymphoplasmacellular infiltrates	36 (90)
Rosette formation	5 (13)
Treatment	
First agent	
Budesonide	1
Prednisone	15
Prednisolone	17
Methylprednisolone	3
Hydrocortisone	1
Steroids	3
No treatment	2
Second agent	
Azathioprine	8
Prednisolone	2
Clinical outcomes	
Time to the first improvement, days (mean ± SD)	4.6 ± 2.9
Time to resolution, days (mean ± SD)	44.6 ± 37
Alive	37
Dead	3

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; IgG: Immunoglobulin G; ANA: Antinuclear antibody; ASMA: Anti-smooth muscle antibody; SLA/LP: Anti-soluble liver antigen/liver-pancreas; AMA: Anti-mitochondrial antibodies; ANCA: Anti-neutrophil cytoplasmic antibodies; Ds-DNA: Anti-double stranded DNA.

Furthermore, increasing evidence highlighted the intrinsic capability of the virus to hyper-stimulate immune response leading to the development of autoimmune disease. However, only two reports identify SARS-CoV-2 as a trigger instrument for developing AILDs, making any correlation not feasible.

Post-vaccine AIH-like syndrome raises worries about a risk of unprecedented immunological side effects, especially in individuals predisposed to autoinflammatory disorders. However, COVID-19 vaccine-induced AIH is extremely uncommon and has an exceptional prognosis. Hepatologists should consider AIH in patients with jaundice or elevated liver enzymes following COVID-19 vaccination to readily initiate further work-up and treatment with steroids.

FOOTNOTES

Author contributions: Sgamato C and Rocco A drafted the manuscript; Compare D, Minieri S, and Marchitto SA participated in the acquisition, analysis, and interpretation of the literature; Maurea S and Nardone G revised the manuscript for important intellectual content; All authors have read and approved the final manuscript.

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MINIREVIEWS

Review of lymphoma in the duodenum: An update of diagnosis and management

Masaya Iwamuro, Takehiro Tanaka, Hiroyuki Okada

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Abstract

The presentation, subtype, and macroscopic images of lymphoma vary depending on the site of the tumor within the gastrointestinal tract. We searched PubMed for publications between January 1, 2012 and October 10, 2022, and retrieved 130 articles relating to duodenal lymphoma. A further 22 articles were added based on the manual screening of relevant articles, yielding 152 articles for full-text review. The most predominant primary duodenal lymphoma was follicular lymphoma. In this review, we provide an update of the diagnosis and management of representative lymphoma subtypes occurring in the duodenum: Follicular lymphoma, diffuse large B-cell lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue, mantle cell lymphoma, and T-cell lymphomas.

Key Words: Diagnosis; Diffuse large B-cell lymphoma; Duodenal neoplasms; Esophagogastroduodenoscopy; Follicular lymphoma; Gastrointestinal lymphoma

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Core Tip: Among cases of primary duodenal lymphoma, follicular lymphoma was the most predominant, followed by diffuse large B-cell lymphoma, extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT lymphoma), mantle cell lymphoma, and T-cell lymphomas. A watch and wait policy is acceptable for follicular lymphoma. Observation without treatment is also an option for MALT lymphoma. However, it should be noted that duodenal MALT lymphoma has a higher rate of transformation to diffuse large B-cell lymphoma than gastric MALT lymphoma. Diffuse large B-cell lymphoma, mantle cell lymphoma, and T-cell lymphomas generally require systemic treatment.

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INTRODUCTION

Gastrointestinal lymphoma is a relatively rare tumor, accounting for 1%–8% of malignant neoplasms of the gastrointestinal tract[1-3]. However, detection and management of gastrointestinal lymphoma lesions is important, as 30%–40% of extranodal lymphomas occur in the gastrointestinal tract[4]. The lymphoma subtype, presentation, and macroscopic images vary depending on the site of the tumor in the gastrointestinal tract. Thus, understanding of the specific features is essential for gastroenterologists to ensure prompt diagnosis and appropriate management. The stomach is the most commonly involved site of lymphomas in the gastrointestinal tract, with the duodenum being less common. Analysis of the United States population based on the Surveillance, Epidemiology, and End Results (SEER) program revealed that the prevalence of primary gastric, small intestinal, and duodenal lymphomas is approximately in the ratio of 10:3:1[5]. Recent studies have found that some subtypes of lymphoma that develop in the duodenum are distinct from those that develop in other parts of the gastrointestinal tract. Here, we review articles describing duodenal lymphoma published within the last 10 years and provide a summary update of contemporary diagnosis and management of the disease.

SEARCH STRATEGY

We searched PubMed for all peer-reviewed articles from January 1, 2012 to October 10, 2022. No study design filters were used. Manual screening of additional relevant articles was performed using a reference list of selected articles meeting eligibility criteria. The search strategy used the keywords 'lymphoma' and 'duodenum'. The search was performed by the lead author (Iwamuro M). Criteria for article inclusion were: (1) Peer-reviewed articles describing cases of duodenal lymphoma; and (2) review articles, original articles, case series, and case reports. Exclusion criteria were: (1) Articles written in languages other than English; (2) animal and cell studies; and (3) the primary topic of study was not duodenal lymphoma. Eligible articles were evaluated in full.

SEARCH RESULTS

Figure 1 shows a flow diagram summarizing the identification, screening, eligibility, and exclusion processes of the literature search. The initial search returned 288 articles. Among these, lymphoma was not the subject in 42 articles. Meanwhile, in 36 articles, case(s) of lymphoma was described but the duodenum was not involved. Articles on animal and cell studies (n = 31), articles in which the primary topic was not duodenal lymphoma (n = 26), and articles written in language other than English (n = 23) were also excluded. After application of the exclusion criteria, 130 articles were retrieved from the initial PubMed search. A further 22 articles were added based on the manual screening of relevant articles. In total, 152 articles were used for full-text review.

Table 1 shows the results of the PubMed database literature search according to the lymphoma subtype. Follicular lymphoma was reported most frequently (48 articles), followed by diffuse large B-cell lymphoma (17 articles), extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) (9 articles), enteropathy-associated T-cell lymphoma (9 articles), mantle cell lymphoma (5 articles), plasmablastic lymphoma (4 articles), monomorphic epitheliotropic intestinal T-cell lymphoma (4 articles), Burkitt lymphoma (4 articles), and anaplastic large cell lymphoma (4 articles). Other rare subtypes of lymphoma occurring in the duodenum reported within the last 10 years were also included.

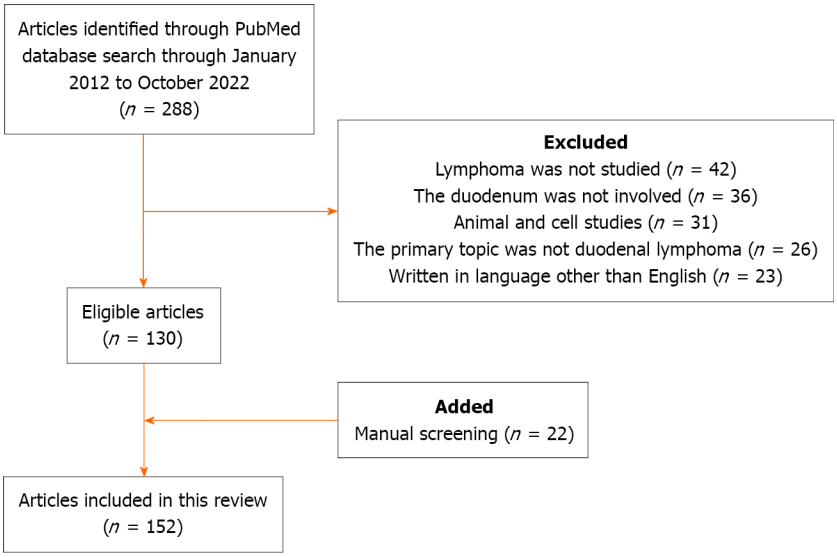
Table 1 Literature search results

Lymphoma subtype	Number of articles
Follicular lymphoma	48[1]
Diffuse large B-cell lymphoma	17[1]
MALT lymphoma	9[1]
Enteropathy-associated T-cell lymphoma	9
Mantle cell lymphoma	5
Plasmablastic lymphoma	4
Monomorphic epitheliotropic intestinal T-cell lymphoma	4
Burkitt lymphoma	4
Anaplastic large cell lymphoma	4
Refractory celiac disease type II	2
Peripheral T-cell lymphoma	2
Marginal zone lymphoma	2
Intravascular Lymphoma	2
NK/T-cell lymphoma	2
Post-transplant lymphoproliferative disease	1
Mycosis fungoides	1
Methotrexate-associated lymphoproliferative disorder	1
Intestinal T-cell lymphoma	1
Immunoproliferative small intestinal disease	1
Extracavitary primary effusion lymphoma	1
Cutaneous large B cell lymphoma	1
Cutaneous aggressive T-cell lymphoma	1
B-lymphoblastic lymphoma	1
Non-Hodgkin lymphoma	1
T cell lymphoma	1
B cell lymphoma	1
Aggressive B-cell lymphoma	1
General review of lymphoma	3
Subtype unspecified	2

[1]One article described follicular lymphoma and MALT lymphoma and another article described follicular lymphoma and diffuse large B-cell lymphoma.
MALT lymphoma: Extranodal marginal zone lymphoma of mucosa associated lymphoid tissue.

PREVALENCE OF LYMPHOMA SUBTYPES IN THE DUODENUM

Analysis of the United States population based on the SEER program revealed a total of 1060 cases of primary duodenal lymphoma identified between 1998 and 2015[5]. Among the primary duodenal lymphoma, the most frequent was follicular lymphoma (41.1%), followed by diffuse large B-cell lymphoma (32.8%), MALT lymphoma (13.8%), mantle cell lymphoma (2.7%), and T-cell lymphoma (2.6%). Meanwhile, diffuse large B-cell lymphoma was the most predominant subtype in the stomach (48.9%) and small intestine (jejunum and ileum, 54.9%), while follicular lymphoma was less frequent in the stomach (2.2%) and small intestine (23.0%). A population-based study in a Japanese prefecture included 350 cases of lymphoma involving the gastrointestinal tract, whether primarily or secondarily [6]. The affected sites were the stomach (62.6%), large intestine (15.4%), small intestine (14.3%), duodenum (6.0%), esophagus (0.3%), and appendix (0.3%). The subtypes of duodenal lymphoma (n = 21) were follicular lymphoma (61.9%), followed by diffuse large B-cell lymphoma (14.3%), MALT lymphoma (14.3%), peripheral T-cell lymphoma (4.8%), and adult T-cell leukemia/lymphoma (4.8%).



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Figure 1 Flow diagram summarizing the identification, screening, eligibility, and exclusion processes of the literature search.

In the following sections, we review recent advances and basic knowledge relating to the diagnosis and management of representative lymphoma subtypes occurring in the duodenum.

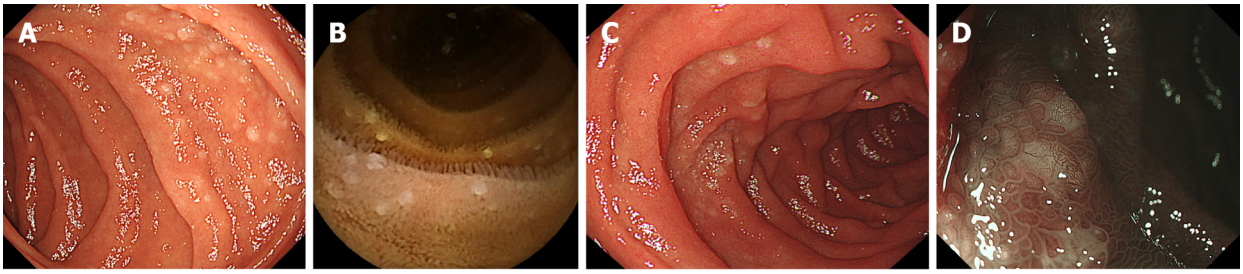
FOLLICULAR LYMPHOMA

As described above, follicular lymphoma is the most common subtype of lymphoma occurring in the duodenum. The majority of cases are asymptomatic and are identified serendipitously during esophagogastroduodenoscopy examinations[7,8]. Follicular lymphomas most frequently occur in the descending part of the duodenum. The typical macroscopic feature is multiple white granules[9] (Figure 2). Magnified observation is useful when it is difficult to distinguish between minute lesions of this and other diseases. Follicular lymphoma is suspected if nodular white submucosal deposits or white villus enlargement are observed by magnified observation[10] (Figure 3). Biopsy reveals small to medium-sized tumor cells that form follicular structures and diffusely invade into the villi. Immunostaining is essential for definitive diagnosis, and CD10, BCL6 and BCL2 are usually positive in follicular lymphoma[7]. In addition, the t(14;18)(q32;q21) translocation of the immunoglobulin heavy chain gene and the BCL2 gene is characteristic of follicular lymphoma. Thus, fluorescence *in situ* hybridization analysis of chromosomal translocation provides complementary information for diagnosis.

Follicular lymphoma is an indolent lymphoma that grows slowly over years. A watch and wait policy is acceptable, provided that appropriate chemotherapy is initiated when the disease progresses and symptoms develop. Comparative study between patients with intestinal follicular lymphoma treated with rituximab-combined chemotherapy (n = 14) and those with watch and wait policy (n = 15) showed comparable outcomes[11-13]. In addition, one prospective study of 31 patients with primary gastrointestinal follicular lymphoma revealed that spontaneous shrinkage or complete disappearance was observed in nine patients[14]. These results confirm the suitability of follicular lymphoma management using the watch and wait policy. Radiation therapy is one option for duodenal lymphomas if the lesion is localized to the field of irradiation[15]. However, duodenal lymphomas are often accompanied by jejunal and/or ileum lesions. Since the gastrointestinal lesions are sometimes negative by positron emission tomography or computed tomography scans[16], evaluation of the whole intestines with colonoscopy and small-intestinal endoscopy is essential when radiotherapy is considered [8]. Recent research has revealed that circumferential location of follicular lesions (more than half of the circumference of the intestinal lumen) and fusion of follicular lesions (dense granular elevations with indistinct boundaries) are significant predictive factors for progression of clinical stage, extension of intestinal lesions, or transformation to diffuse large B-cell lymphoma[17]. Patients with these endoscopic features may require surveillance in the short term.

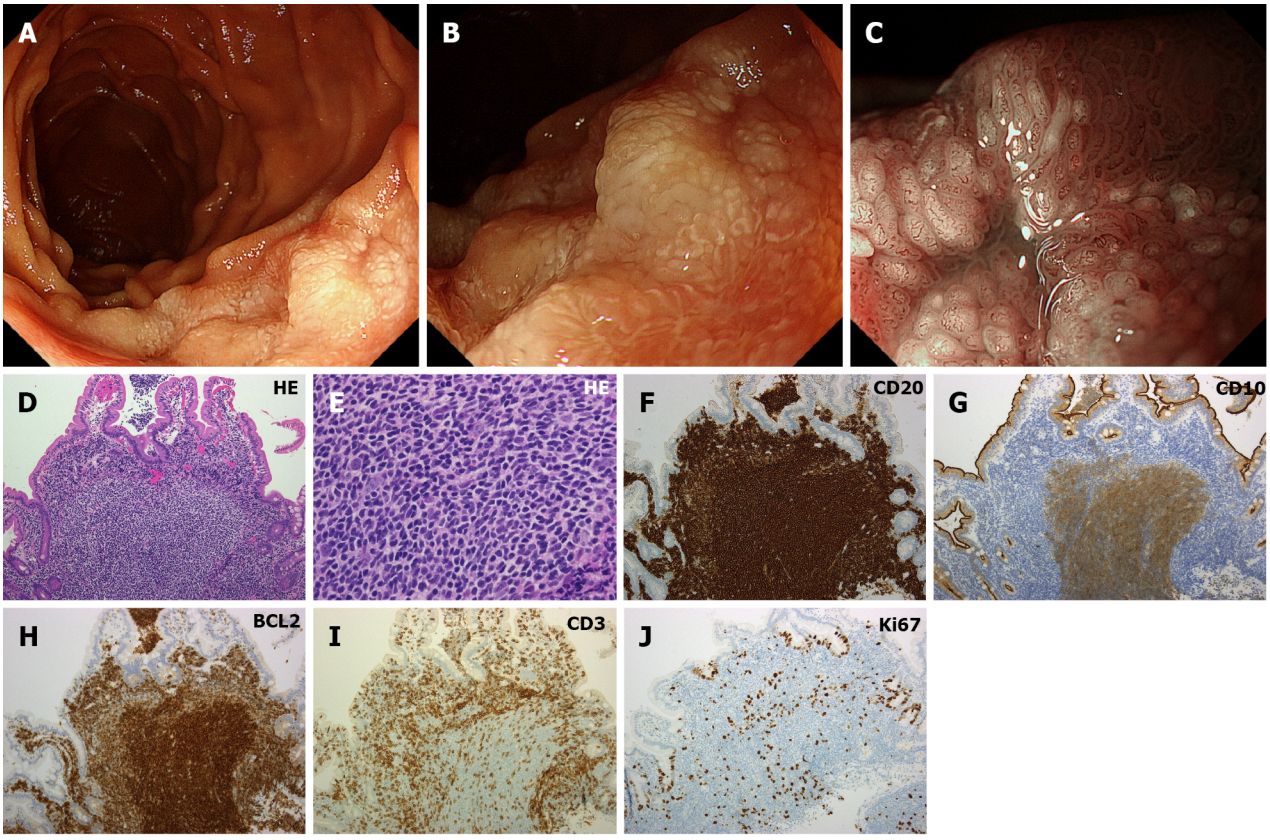
DIFFUSE LARGE B-CELL LYMPHOMA

Diffuse large B-cell lymphoma can occur in any part of the gastrointestinal tract, but it is often seen in



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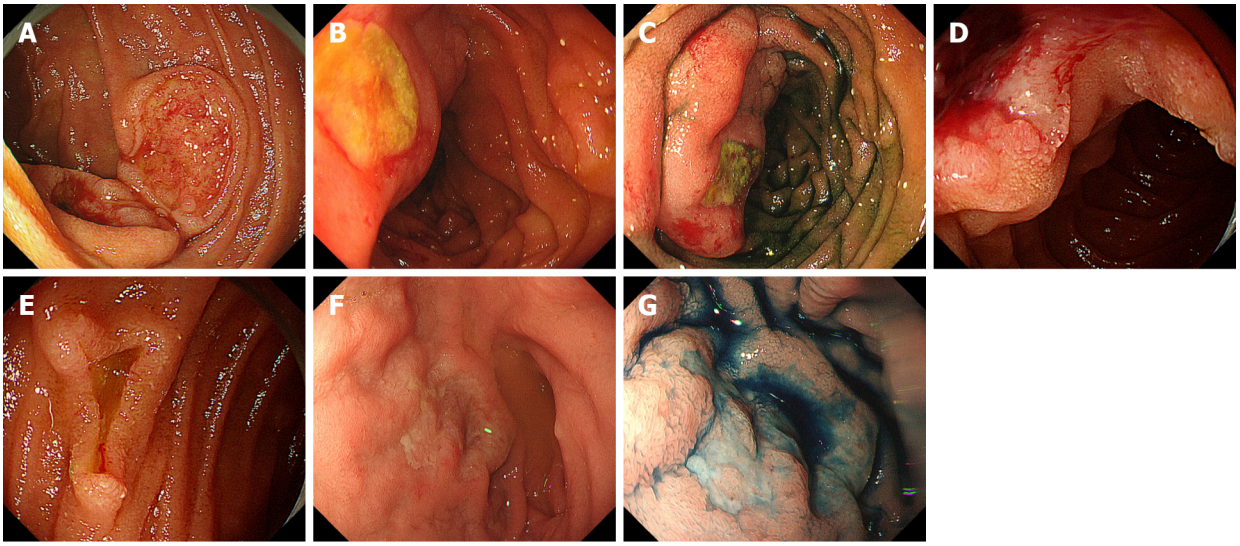
Figure 2 Representative endoscopic images of duodenal follicular lymphoma (Cases 1 and 2). A: Case 1. Esophagogastroduodenoscopy reveals typical feature of follicular lymphoma showing multiple whitish granules in the descending portion of the duodenum; B: Case 1. Video capsule enteroscopy shows follicular lymphoma lesions in the jejunum; C: Case 2. Another case of duodenal follicular lymphoma; D: Case 2. Magnifying observation with narrow band imaging shows white deposits.



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Figure 3 Representative endoscopic and pathological images of duodenal follicular lymphoma (Case 3). A: Esophagogastroduodenoscopy shows thick, white mucosa in the descending portion of the duodenum; B: Magnifying observation reveals enlarged whitish villi; C: Magnifying observation with narrow band imaging emphasizes enlarged villi with elongated vasculature on the surface; D–J: Pathological images of the biopsied specimen. Hematoxylin and eosin stain shows medium-sized tumor cells that form follicular structures and diffusely invade into the villi (D: × 10, E: × 40). Lymphoma cells are positive for CD20, CD10, and BCL2, while negative for CD3. Tumor cells are sparsely positive for Ki67. HE: Hematoxylin and eosin.

the stomach and ileocecal region, and duodenal involvement is rare[18]. Retrospective analysis of 126 patients with intestinal diffuse large B-cell lymphomas revealed that the ileocecal region was the most commonly involved (50.0%), followed by the small intestine (*i.e.*, jejunum and/or ileum, 23.0%), duodenum (18.3%), colon (11.1%), and rectum (5.6%)[19]. Diffuse large B-cell lymphoma lesions in the duodenum and large intestine tend to be secondarily involved, while those in the ileocecal region and small intestine are *de novo* neoplasms found in limited-stage disease[19]. It has also been reported that in approximately 30% of cases, duodenal diffuse large B-cell lymphoma are accompanied by gastric lesions [19]. On esophagogastroduodenoscopy, ulcerative and protruded lesions are typical morphology of duodenal diffuse large B-cell lymphoma[20]. Particularly, an auriculate ulcer mound is characteristic of diffuse large B-cell lymphoma[21] (Figure 4), although it is rarely seen in other lymphomas and cancers. Biopsies tend to consist of a diffuse proliferation of large B cells, with Ki-67 positivity generally greater



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Figure 4 Representative endoscopic images of diffuse large B-cell lymphoma (Cases 4–7). A: Case 4. Esophagogastroduodenoscopy reveals duodenal lesions showing auriculate ulcer mounds; B and C: Case 5. A protruded, ulcerative lesion is seen in the duodenum; C: After indigo carmine spraying; D and E: Case 6. Multiple ulcerative lesions in the duodenum; F and G: Case 7. Irregular-shaped, shallow ulcers in the duodenum.

than 40%.

Diffuse large B-cell lymphoma is an aggressive (intermediate-grade) lymphoma that progresses on a monthly basis. However, this disease often responds well to treatment and long-term remission can be expected. Neoplastic cells of diffuse large B-cell lymphoma often exist through the entire thickness of the gastrointestinal tract[18]. Thus, patients should be counseled on the risk of intestinal perforation, bleeding, and stricture after the initiation of chemotherapy[22].

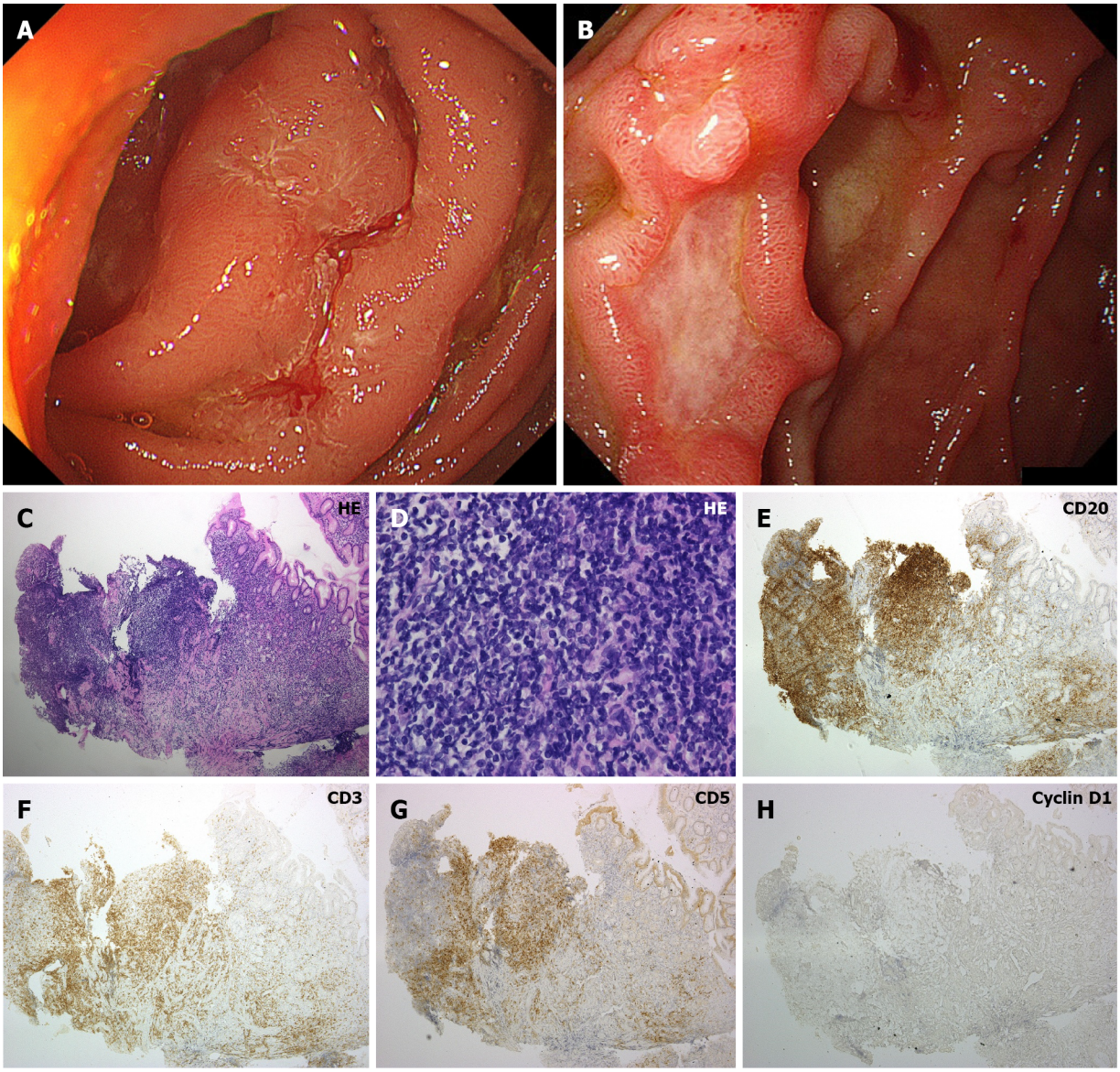
MALT LYMPHOMA

MALT lymphoma is common in the stomach and occurs less frequently in the duodenum. Duodenal MALT lymphoma presents various macroscopic features (Figure 5). According to reports describing 13 cases of duodenal MALT lymphoma, nodular lesions were the predominant feature (58.3%), followed by ulcer (16.7%), flat depression (16.7%), and subepithelial tumor (8.3%)[23]. Elevated lesions have also been described in case reports[24,25]. Among these, nodular lesions showing white granular protrusions must be differentiated from follicular lymphoma[23,26]. Endoscopic biopsy specimen predominantly shows small to medium-sized lymphoma cells, mixed with varying proportions of large cells. Lymphoepithelial lesions are infrequently observed in the duodenal MALT lymphoma[27]. Immunostaining is essential for definitive diagnosis and differentiation from other B-cell lymphomas.

Although some of the duodenal MALT lymphoma lesions reportedly regress with *H. pylori* eradication therapy, the response rate is lower than that for gastric MALT lymphoma[23]. Radiation therapy is indicated for limited-stage disease in which the disease is confined to the duodenum or limited to the regional lymph nodes[28,29]. Systemic chemotherapy is administered for non-limited-stage disease. Observation without treatment is an option because MALT lymphoma generally spreads slowly, but it should be noted that duodenal MALT lymphoma has a higher rate of transformation to diffuse large B-cell lymphoma than gastric MALT lymphoma[23].

MANTLE CELL LYMPHOMA

Mantle cell lymphoma forms multiple polypoid elevations throughout the gastrointestinal tract including the duodenum, and has been termed multiple lymphomatous polyposis[30-32] (Figure 6). Multiple lymphomatous polyposis is a typical intestinal lesion of mantle cell lymphoma. However, although infrequently, other lymphomas can also present with this feature. We reviewed 35 cases of mantle cell lymphoma with gastrointestinal involvement and the involved sites were the stomach (74.3%), colon (57.1%), ileum (47.6%), rectum (47.6%), duodenum (34.3%), cecum (14.3%), and esophagus (5.7%)[33]. Mantle cell lymphoma lesions in the intestines (from duodenum to rectum, n = 22) predominantly showed multiple lymphomatous polyposis (10 or more elevated lesions, 77.3%), followed by protruded (1–9 elevated lesions, 18.2%) and superficial lesions (4.5%). The duodenal lesions



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Figure 5 Representative endoscopic and pathological images of duodenal MALT lymphoma (Cases 8 and 9). A: Case 8. A protruded lesion mimicking hypertrophic folds is observed in the duodenum; B: Case 9. Esophagogastroduodenoscopy shows ulcerative tumor in the duodenum; C–H: Pathological images of Case 9. On hematoxylin and eosin stain, small to medium-sized lymphoma cells are predominant (C: × 10, D: × 40). Neoplastic cells are positive for CD20, while negative for CD3, CD5, and Cyclin D1. HE: Hematoxylin and eosin.

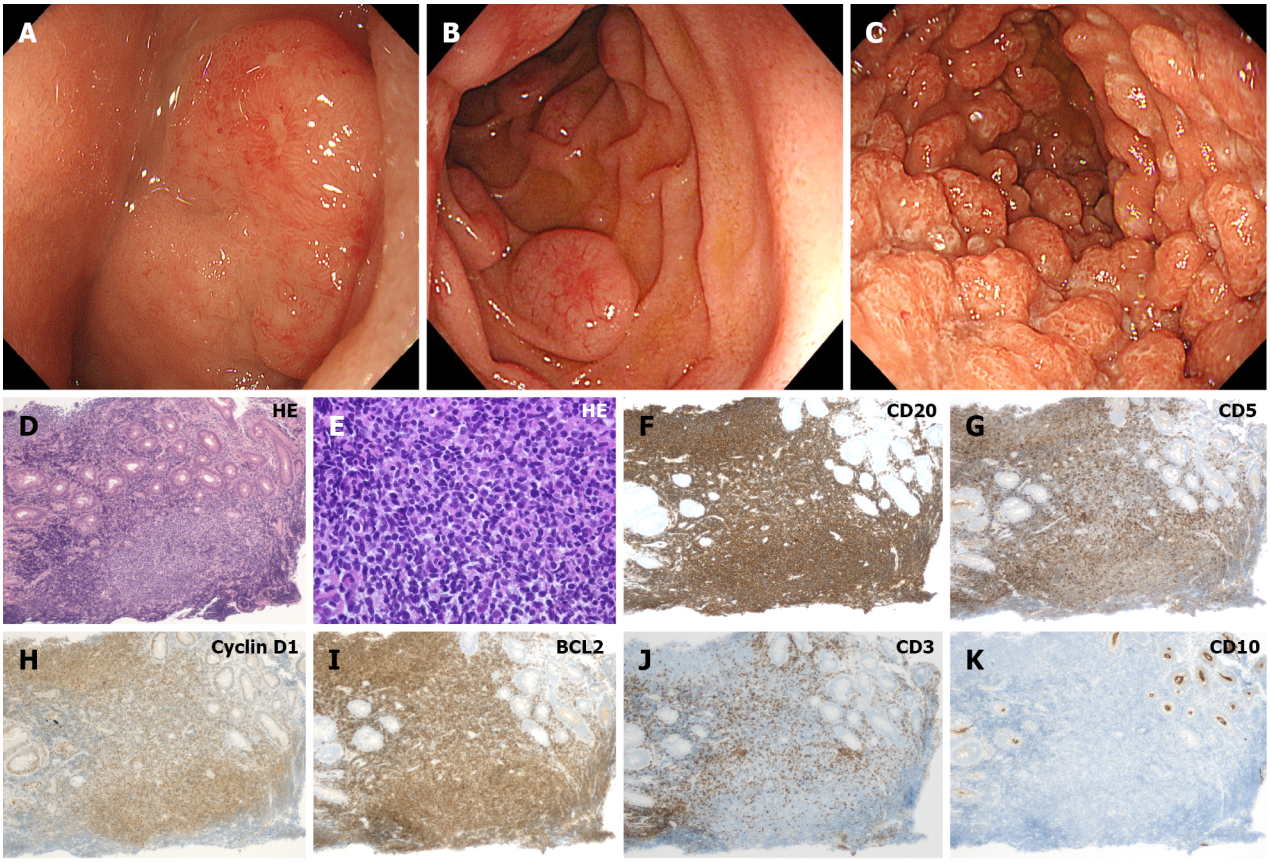
of mantle cell lymphoma often accompany erosion of the tumorous nodules[30,34]. Endoscopic biopsy specimens consist of a homogeneous growth of small to medium-sized lymphoma cells with loose nodular structures in a diffuse pattern. As for other lymphomas, immunostaining is essential for definitive diagnosis. CD5, Cyclin D1, and SOX11 are often positive in mantle cell lymphoma[35].

Despite intensive chemotherapy regimens, mantle cell lymphoma generally remains incurable and is treatment-resistant with multiple relapses. Thus, prompt consultation with hematologists is required after the diagnosis[36].

T-CELL LYMPHOMAS

In the latest version of WHO classification of hematolymphoid tumors of the digestive system, T-cell lymphomas have been classified under four major types: Enteropathy-associated T-cell lymphoma, monomorphic epitheliotropic intestinal T-cell lymphoma, intestinal T-cell lymphoma not otherwise specified (NOS), and indolent T-cell lymphoproliferative disorder of the gastrointestinal tract[36].

Enteropathy-associated T-cell lymphoma, formerly known as type I enteropathy-associated T-cell lymphoma, is the most prevalent among primary intestinal T-cell neoplasms. Lymphoma cells derive



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Figure 6 Representative endoscopic and pathological images of duodenal mantle cell lymphoma (Cases 10–12). A: Case 10. Elevated lesion with erosions on the surface in the duodenum; B: Case 11. Typical morphology of mantle cell lymphoma showing multiple lymphomatous polyposis in the duodenum. Subepithelial lesion-like protruded lesions accompanying dilated vasculature on the surface are seen; C: Case 12. Numerous, diffuse polypoid lesions in the duodenum; D–K: Pathological images of Case 12. Lymphoma cells retain a homogeneous pattern of cell size on hematoxylin and eosin stain (D: × 10, E: × 40). Cells are positive for CD20, CD5, Cyclin D1, BCL2, while negative for CD3 and CD10. HE: Hematoxylin and eosin.

from intraepithelial lymphocytes in celiac disease patients[37,38]. In particular, 30%–52% of refractory celiac disease type II transforms into an enteropathy-associated T-cell lymphoma within five years[39]. Monomorphic epitheliotropic intestinal T-cell lymphoma, formerly known as type II enteropathy-associated T-cell lymphoma, derives from intraepithelial T lymphocytes and is typically not linked to celiac disease[40-44]. Aggressive T-cell lymphoma that lacks the clinical and pathological features of enteropathy-associated T-cell lymphoma, monomorphic epitheliotropic intestinal T-cell lymphoma, anaplastic large cell lymphoma, or extranodal NK/T-cell lymphoma is now categorized as intestinal T-cell lymphoma NOS[45]. Indolent T-cell lymphoproliferative disorder of the gastrointestinal tract represents clonal proliferation of T lymphocytes within the lamina propria, most commonly in the small intestine and colon[46]. The prognosis of T-cell lymphomas is generally poor, except for indolent T-cell lymphoproliferative disorder that shows prolonged survival with persistent, chronic relapsing disease.

CONCLUSION

We reviewed relevant articles mainly published within the last 10 years associated with duodenal lymphomas and summarized updates of representative lymphoma subtypes. Though there is no doubt that endoscopic examinations play a major role in the diagnosis of duodenal lymphoma, understanding of each pathological subtype will improve the diagnosis and initial treatment response to the disease. We believe this review will be helpful for gastroenterologists and endoscopists who diagnose and treat patients with duodenal lymphoma.

FOOTNOTES

Author contributions: Iwamuro M designed the research study and wrote the paper; Tanaka T critically reviewed the

manuscript for important intellectual content; Okada H approved the manuscript.

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MINIREVIEWS

Comprehensive review on endoscopic ultrasound-guided tissue acquisition techniques for solid pancreatic tumor

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Abstract

Pancreatic ductal adenocarcinoma is speculated to become the second leading cause of cancer-related mortality by 2030, a high mortality rate considering the number of cases. Surgery and chemotherapy are the main treatment options, but they are burdensome for patients. A clear histological diagnosis is needed to determine a treatment plan, and endoscopic ultrasound (EUS)-guided tissue acquisition (TA) is a suitable technique that does not worsen the cancer-specific prognosis even for lesions at risk of needle tract seeding. With the development of personalized medicine and precision treatment, there has been an increasing demand to increase cell counts and collect specimens while preserving tissue structure, leading to the development of the fine-needle biopsy (FNB) needle. EUS-FNB is rapidly replacing EUS-guided fine-needle aspiration (FNA) as the procedure of choice for EUS-TA of pancreatic cancer. However, EUS-FNA is sometimes necessary where the FNB needle cannot penetrate small hard lesions, so it is important clinicians are familiar with both. Given these recent developments, we present an up-to-date review of the role of EUS-TA in pancreatic cancer. Particularly, technical aspects, such as needle caliber, negative pressure, and puncture methods, for obtaining an adequate specimen in EUS-TA are discussed.

Key Words: Endoscopic ultrasound-guided fine needle biopsy; Endoscopic ultrasound-guided tissue acquisition; Personalized medicine; Genomic profiling test; Pancreatic cancer; Puncture procedure

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Core Tip: Endoscopic ultrasound (EUS)-guided tissue acquisition (TA) began in 1992 as EUS-guided fine-needle aspiration (FNA). Recently, with the development of personalized medicine and precision treatment, the fine-needle biopsy (FNB) needle was developed. EUS-FNB is rapidly replacing EUS-FNA for pancreatic cancer. The EUS-TA strategy with three or more punctures, the stylet retraction method, the torque or fanning technique, and a 22-G or thicker FNB needle may be effective in patients with solid pancreatic tumors scheduled for treatment, including personalized medicine. It is also important clinicians are familiar with both procedures, as EUS-FNA is occasionally necessary when FNB is unsuccessful.

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INTRODUCTION

Approximately 80%-85% of patients with pancreatic cancer are borderline-resectable (BR) requiring neoadjuvant treatment and unresectable with metastases. International guidelines for pancreatic neoplasms recommend pathological diagnosis of pancreatic adenocarcinoma prior to chemotherapy. Therefore, endoscopic ultrasound (EUS)-guided tissue acquisition (TA) is usually performed in patients with BR requiring neoadjuvant therapy, or in metastatic disease requiring palliative therapy[1]. The remaining 15%-20% of patients may benefit from EUS-TA prior to surgery; this can confirm pancreatic neoplasms, and reduce the number of unnecessary operations for other similar conditions (*e.g.*, autoimmune pancreatitis, local chronic pancreatitis, and lymphomas). Before the development of EUS-TA, 5%-10% of patients were misdiagnosed with pancreatic cancer and underwent surgery for pancreatic lesions[2-4].

EUS-TA can be divided into EUS-fine-needle aspiration (FNA) using Menghini needles, and EUS-fine-needle biopsy (FNB) using Franseen or fork-tip needles. FNB has equal or better diagnostic accuracy than FNA, with increased tissue volume and decreased number of punctures for diagnosis[5-8]. Moreover, EUS-FNB may provide higher diagnostic yield for peri-hepatic lymph nodes[9]. FNB increases the number of cases for which ancillary tests such as immunohistochemistry and tumor molecular profiling can be performed[9,10]. Therefore, with the advent of personalized medicine and precision therapy, EUS-FNB is rapidly becoming the procedure of choice for EUS-TA of pancreatic cancer. However, EUS-FNA may be used where the FNB needle cannot penetrate small hard lesions; therefore, there is a need for familiarity with both procedures. Conti *et al*[11] also recommend EUS-FNA and FNB in difficult cases of puncture and sampling. Needle size, the negative pressure applied to the needle, and puncture techniques are important for obtaining an adequate specimen in EUS-TA.

Considering recent developments, we present an up-to-date review of the role of EUS-TA in pancreatic cancer. In particular, technical aspects and novel applications for obtaining appropriate specimens during EUS-TA are discussed.

EUS-FNA

EUS-FNA has 87%-92% sensitivity and 96%-98% specificity for the pathological diagnosis of solid pancreatic malignancy[12-15]. FNA needles with calibers ranging from 19-gauge (G)–25G are available, with many studies comparing the different calibers and technical aspects of the EUS-FNA technique. We present a table summarizing the respective characteristics of FNA and FNB, focusing on key points (Table 1).

FNA needle caliber

The small 25G FNA needle may have a technical advantage over larger bore needles when sampling very fibrous, solid lesions. Moreover, its superior flexibility improves operability, especially when sampling pancreatic head and apex masses from the duodenum[1]. Three meta-analyses compared 25G FNA needles with 22G FNA needles. One of them showed increased diagnostic sensitivity (with similar specificity), while the other two showed no significant differences. Therefore, both calibers (25G and 22G needles) can be used when sampling solid pancreatic lesions[16]. Compared with thinner needles, 19G FNA needles are stiffer and harder to manipulate, increasing technical failure when sampling

Table 1 The respective characteristics of fine-needle aspiration and fine-needle biopsy

	EUS-FNA	EUS-FNB
Recommended needle caliber	22-25G	22G or thicker
Recommended negative pressure	Suction or wet suction	Stylet retraction
Recommended puncture technique	Fanning or DKM	Fanning or torque
Recommended number of needle passes	1-3 times for histological diagnosis, depending on site and size	Three times for NGS
Diagnostic accuracy		FNB ≥ FNA
Tissue volume		FNB ≥ FNA
Suitability for NGS		FNB ≥ FNA
MOSE	Recommended for use in combination	Recommended for use in combination
Complications	Rare	Rare
Cost-effectiveness		FNB ≥ FNA

DKM: Door knocking method; G: Gauge; EUS: Endoscopic ultrasound; FNA: Fine-needle aspiration; FNB: Fine-needle biopsy; MOSE: Macroscopic on-site evaluation; NGS: Next-generation sequencing.

pancreatic head lesions[17].

Negative pressure during EUS-FNA

The conventional aspiration method, where 10 mL of negative pressure is applied using a syringe attached to a needle, is recommended because of its higher sensitivity and diagnostic rate[18,19]. In addition, EUS-FNA with 50 mL negative pressure is superior to 10 mL during tissue collection[20]. The wet aspiration method involves precleaning the needle with saline solution, replacing the air column with liquid, and applying negative pressure. Theoretically, this method improves the cell count and sample quality compared with the conventional aspiration and suction methods, as the negative pressure applied from the syringe is better transmitted to the needle tip[21]. In the stylet-throw-pull method, the stylet is slowly and gradually removed (without using a syringe) while the needle is moved within the lesion to create minimal negative pressure. The diagnostic results for solid pancreatic lesions sampled using stylet-throw-pull method are comparable to the conventional aspiration method[22,23].

EUS-FNA puncture technique

The fanning technique is used to collect specimens from multiple sites with a single puncture by fanning the needle back and forth using the elevator of the endoscope and the up/down dial control. The fanning technique improves diagnostic yield, especially in cancerous tumors with necrotic centers. In a study of pancreatic masses, fanning reduced the number of passes required for diagnosis and resulted in a high first-pass diagnostic rate[24]. The door knocking method (DKM), in which the needle is moved forward within the mass so quickly and powerfully that a sound is emitted when a part of the handle hits the needle stopper, did not improve the accuracy of histological diagnosis. However, it enabled the acquisition of a larger amount of tissue specimen compared with the conventional puncture[25]. Thus, DKM may be advantageous for genomic profiling tests; however, to our knowledge, no study has verified this.

Uehara *et al*[26] reported the optimal number of needle passes for accuracy of histological diagnosis: 3 where the head was < 15 mm; 2 where the head was ≥ 15 mm; 2 where the body and tail were ≤ 15 mm; and 1 where the body and tail were ≥ 15 mm. In total, 93% of pancreatic lesions were correctly diagnosed using these numbers of passes. In addition, size of ≤ 15 mm, head location, and neuroendocrine tumors independently required 2 or more needle passes[26].

EUS-FNB FOR HISTOLOGICAL DIAGNOSIS

The desire to collect samples with increased cell counts and preserved tissue structures led to the development of a new type of needle, the FNB needle. This needle is specifically designed to collect core tissue samples. To date, three generations of FNB needles exist. The first generation, the Tru-Cut 19G needle (QuickCore, Cook Endoscopy, Limerick, Ireland), became rapidly outdated due to inflexibility and technical problems[27]. In a randomized controlled trial (RCT) comparing a 19G Tru-Cut needle with a conventional 22G FNA needle in transduodenal EUS-TA of pancreatic head lesions, Sakamoto *et*

al[28] reported that the 22G FNA needle group had significantly higher diagnostic accuracy for malignancy and technical success rate than the 19G Tru-Cut needle group. The second generation of FNB needles, Procore needles (Cook Endoscopy, Limerick, Ireland), were created with a reverse bevel design. More than 10 years after the Tru-Cut needle was introduced, the third generation EUS-FNB needles for core biopsy were developed. They could collect more tissue than the conventional FNA needle, thus improving diagnostic results. Various types of needles have been developed, including the fork type (SharkCore; Medtronic, Minneapolis, MN, United States), flanged type (Acquire; Boston Scientific, Marlborough, MA, United States) (Top-gain; Medi-Globe, Achenmühle, Germany), and 20G FNB needles with forward-facing core traps (ProCore 20G; Cook Medical)[27] (Figure 1). A meta-analysis demonstrated the diagnostic performance of the new generation FNB needles, namely the crown and fork tip needles. They showed a remarkable diagnostic accuracy of 96% for solid pancreatic lesions, with no significant difference between the crown and fork-tip needles (97% and 95%, respectively, $P = 0.8$)[29]. Renelus *et al*[7] conducted a meta-analysis of RCTs published between 2012 and 2019; they found that FNA demonstrated significantly reduced diagnostic accuracy compared with FNB (81% and 87%, respectively, $P = 0.005$). In addition, FNA required an increased number of mean passes compared with FNB (2.3 and 1.6, respectively, $P < 0.0001$). Furthermore, there was no significant difference in the rate of adverse events between FNA and FNB needles (1.8% and 2.3%, respectively, $P = 0.64$)[7].

An RCT analysing cost-effectiveness found that pancreatic mass EUS-FNB (two passes without on-site cytopathology evaluation) was more cost-effective than EUS-FNA (number of passes dictated by on-site cytopathology evaluation). Variables with the largest impact were EUS procedure and sedation cost, specimen adequacy, and diagnostic yield associated with EUS-FNB[30].

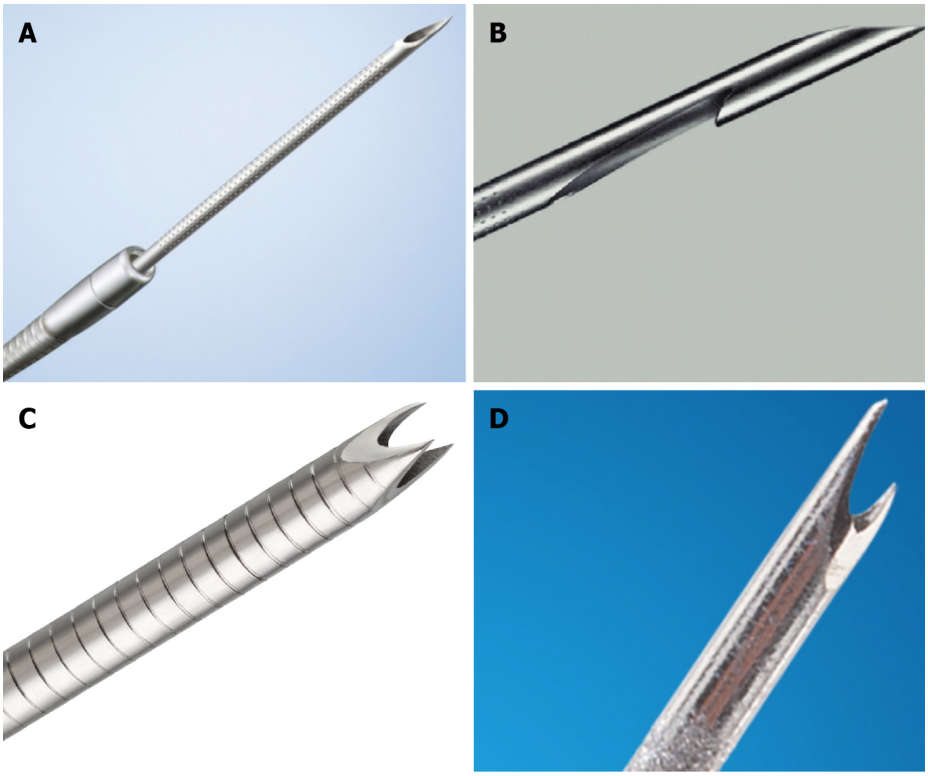
THE ERA OF PERSONALIZED AND PRECISION MEDICINE

Several studies have reported increased tissue volume with EUS-FNB samples compared with EUS-FNA. One study reported a 20-fold increase in tissue volume[9,31,32]. Elhanafi *et al*[10] reported that FNB resulted in a higher proportion of sufficient samples for targeted next-generation sequencing (NGS) than FNA (90.9% *vs* 66.9%; $P = 0.02$). In multivariable modeling, only FNB [odds ratio = 4.95, 95% confidence interval (CI): 1.11-22.05, and $P = 0.04$] was associated with sufficient sampling capacity for genomic testing[10].

Pancreatic ductal adenocarcinoma is anticipated to become the second leading cause of cancer-related mortality by 2030, with high mortality considering the incidence. Given the low median survival time of 12 mo for patients with advanced metastatic pancreatic cancer (ductal adenocarcinoma in particular)[33,34], urgent new treatments are warranted. Molecular profiling data has recently revealed that up to 25% of pancreatic cancers have actionable molecular changes[35-42], defined as changes with strong clinical or preclinical evidence suggesting specific treatment efficacy[34]. Actionable molecular changes were found in 5.5%-21.7% of EUS-FNA/B specimens from pancreatic ductal adenocarcinoma patients [39,43]. Pishvaian *et al*[34] reported that patients with actionable molecular alterations treated with matched therapy ($n = 46$) had significantly longer median overall survival times than those treated with unmatched therapies [$n = 143$; 2.58 years *vs* 1.51 years; hazard ratio (HR) 0.42 (95%CI: 0.26-0.68), $P < 0.001$][34]. Therefore, EUS-TA can improve diagnostic accuracy and help ensure successful personalized medicine, with EUS-FNB expected to be particularly useful.

Advances have been made in both tissue collection methods and devices, and genetic analysis technology. The most common genomic alterations noted in metastatic pancreatic cancer specimens are in the *KRAS, TP53, CDKN2A,* and*SMAD4* genes. The first genetic analysis reported using EUS-FNA samples was KRAS mutation analysis. This driver mutation is found in more than 90% of pancreatic cancers, and drugs targeting KRAS mutations, such as Sotrasib, may be effective for pancreatic cancer [27,44]. The DNA mismatch repair (MMR) pathway is an important function that identifies and corrects base pair mismatches in DNA. Loss of MMR function leads to elevated microsatellite instability (MSI)-high, making the MSI test useful for evaluating pembrolizumab response. Sugimoto *et al*[45] reported that in unresectable pancreatic ductal adenocarcinoma, EUS-FNB with the Franseen 22G needle had a higher success rate in MSI analysis than EUS-FNA [FNB, 88.9% (8/9) *vs* FNA, 35.7% (5/14); $P = 0.03$].

Targeted genome sequencing (TGS) applies polymerase chain reaction technology to construct and sequence a library of specific regions. Currently available multigene NGS systems, such as MSK-IMPACT (Memorial Sloan Kettering Cancer Center, New York, NY, United States) and FoundationOne CDx (F1CDx; Foundation Medicine, Cambridge, MA, United States), screen several hundred cancer-related genes simultaneously as companion diagnostic tests[46,47]. Whole genome sequencing (WGS) can detect various genomic structural alterations (translocations, inversions, duplications, chromosomal aberrations, chromosomal breaks) by obtaining sequence information of the entire genome, including non-coding regions. WGS may be more useful than TGS, but it is significantly more costly, requires more samples, and its superiority in therapeutic selection has not yet been proven[48]. RNA sequencing is a newly developed technology that can identify fusion genes faster, with greater sensitivity, and more efficiently than DNA panels. It can also detect new genes and genetic mutations.



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Figure 1 Examples of needle designs. A: Menghini needle (EZ shot 3, Olympus medical systems, Tokyo, Japan); B: Reverse-beveled needle (ProCore, Cook Medical, Bloomington, IN, United States); C: Franseen needle (AcquireTM, Boston Scientific, Marlborough, MA, United States); D: Fork-tip needle (SharkCore, Medtronic, Minneapolis, MN, United States).

Agents for actionable molecular changes are still being investigated. For example, evidence suggests trametinib in GNAS alterations[49], sotorasib in KRAS G12C alterations[44], olaparib in germline *BRCA1/2* mutation[50], entrectinib in *NTRK* gene fusion[51], pembrolizumab in MSI-high[52,53], and afatinib in NRG1 fusions[54,55] may be effective treatment options. In malignancies such as pancreatic cancer, it is likely that multiple genomic drivers may be present. A combination of compatible drugs with targeting multiple genomic alterations at once may overcome this; however, evidence is lacking, and further studies are needed[56,57]. The application of precision medicine for treating pancreatic ductal adenocarcinoma has just begun, and further development of genetic testing equipment, drugs, and tissue collection devices is required[27].

EUS-FNB FOR THE ERA OF PERSONALIZED AND PRECISION MEDICINE

FNB needle type and caliber

Since EUS-FNB is more suitable than EUS-FNA for personalized medicine, further investigation into needle size and type has been conducted. The abovementioned meta-analysis evaluating crown and fork-tip needles, showed equivalent sample adequacy, diagnostic accuracy, optimal histological core procurement, mean number of needle passes, pooled specificity, and sensitivity of these two FNB needles[29]. Oh *et al*[58] compared 22G with 25G Franseen needles in the three needle passes technique, with suction using 10 mL syringe, and movement within the target lesion at least 10 times using the fanning technique. They reported no diagnostic accuracy difference between the two needles; however, the 22G Franseen needle was superior to the 25G needle in histologic core facilitated easier collection of appropriate specimens for the OncoGuide NCC Oncopanel System [70.0% (49/70) *vs* 28.6% (20/70), respectively; $P < 0.001$]. The 19G FNB needle was reported to have the potential for easier collection of appropriate specimens for the OncoGuide NCC Oncopanel System compared with the 22G FNB needle, however, concerns were expressed about risks such as bleeding and pancreatic juice leakage[59,60]. For successful personalized medicine, 22G FNB needles are better than 25G ones. However, usage of 19G FNB needles raises concerns regarding accidental injury and harder to manipulate compared with 22G ones.

Negative pressure during EUS-FNB

A randomized controlled trial of tissue samples from patients with pancreatic masses on imaging compared collection techniques using Menghini-tip, reverse-bevel, Franseen, and fork-tip needles. A second randomized control, patient-by-patient analysis, compared the use of suction, no suction, and stylet retraction during biopsies. The biopsy samples collected by fork-tip or Franseen needles had significantly higher cellularity than those collected by Menghini-tip needles or reverse-bevels (P < 0.001). The predictors of high cellularity for pancreatic masses showed that Franseen needles, fork-tip needles, not applying suction, stylet retraction, and pancreatic mass size of > 3 cm were associated with high cellularity. Therefore, they recommended using a negative pressure technique with every EUS-TA needle for an optimal outcome, as shown in Table 2[61]. Stylet retraction and standard suction technique may be equivalent when using the 20G reverse-beveled needle[62]; however, the stylet retraction technique is considered equally or more useful than the standard suction technique when performing EUS-FNB.

EUS-FNB puncture technique

Sample quality scores were significantly higher when obtained by the torque and fanning techniques rather than the standard one (P < 0.001)[63]. The new torque technique consists of turning the echoendoscope counterclockwise or clockwise while repeated to-and-fro movements are performed to reach multiple areas within the lesion; this is obtained by maneuvering the "up-down" articulating dial of the echoendoscope. Conversely, in the standard technique, the mass is pierced by the tip of the needle in a unidirectional movement. Studies on FNA needles suggest that the DKM may be useful for increasing cell volume[25]; however, there are no specific studies on DKM in FNB.

The European Society of Gastrointestinal Endoscopy recommends that 2-3 needle passes are sufficient to ensure a sensitivity of at least 90% for the diagnosis of malignancy[16]. However, evidence on the number of needle passes required for successful personalized medicine is lacking. In a few recent retrospective studies, a median number of 3 FNB needle passes (interquartile range 3-4) yielded sufficient tissue for targeted NGS in 91% of patients[10].

Based on these findings, we believe that the EUS-TA strategy with three or more punctures, the stylet retraction method, the torque or fanning technique, and a 22-G or thicker FNB needle may be effective in patients with solid pancreatic tumors scheduled for treatment, including personalized medicine.

RAPID ON-SITE PATHOLOGIST EVALUATION AND MACROSCOPIC ON-SITE QUALITY EVALUATION

It is unclear whether an on-site pathologist improves the diagnostic accuracy of EUS-FNA for solid pancreatic lesions; however, Rapid On-Site Pathologist Evaluation (ROSE) can reduce the number of needle passes required to obtain a pathological diagnosis[64,65]. The subsequent introduction of the FNB needle has made it possible to collect large quantities of macroscopic white samples. A recent global, randomized trial showed that ROSE did not affect the diagnostic accuracy of EUS-FNB (96.4% with ROSE *vs* 97.4% without ROSE, P = 0.396)[66]. In addition, the application of ROSE is limited by the availability and additional cost of trained cytopathologists at each facility; therefore, ROSE is unlikely to be recommended for future diagnostic practice[67].

Direct observation of specimens obtained by FNA/B, macroscopic on-site evaluation (MOSE), is more feasible and readily available alternative to ROSE[68,69]. Kaneko *et al*[70] showed that the macroscopic visible core (MVC) length and histological sample quantity were positively correlated in EUS-FNB using a 22G Franseen needle. Multivariate analysis showed that MVC length ≥ 30 mm on MOSE was a significant factor affecting suitability for NGS (odds ratio 6.19; 95%CI: 2.72-14.10).

NEEDLE TRACT SEEDING

Theoretically, cancer seeding through the needle tract may occur more frequently in the body or tail of the lesion when using the transgastric approach, than in the head of the lesion using the transduodenal approach[71]. This is because the EUS-FNA tract of pancreatic head cancers is later resected in a blocked fashion along with the pancreatic head in curative surgery. In contrast, the EUS-FNA tract of lesions in the body or tail lies beyond the surgical resection margin[72,73].

Ngamruengphong *et al*[73] reported that EUS-FNA for pancreatic head, body, and tail cancers was marginally associated with improved overall survival (HR 0.84, 95%CI: 0.72-0.99) but did not affect cancer-specific survival (HR 0.87, 95%CI: 0.74-1.03). Park *et al*[74] reported 528 patients with distal pancreatic cancer who underwent distal pancreatectomy. Among these, 193 were treated using EUS-FNA, and 335 were not. The between-group recurrence rates were comparable (EUS-FNA, 72.7%; non-EUS-FNA, 75%; P = 0.58) at follow-up (median, 21.7 mo), with similar cancer-free survival (P = 0.58), showing that preoperative EUS-FNA does not reduce cancer-specific or overall survival[74]. However,

Table 2 Recommended negative pressure procedural technique for each endoscopic ultrasound-guided tissue acquisition needle to achieve optimal outcomes[61]

Needle type	Best cellularity	Best diagnostic accuracy	Best overall outcome (specimen)
Menghini	Stylet retraction = suction = no suction	Suction	Suction
Reverse-bevel	Stylet retraction = suction	Suction	Suction
Franseen	Stylet retraction	Stylet retraction = suction	Stylet retraction
Fork-tip	Stylet retraction = no suction	Stylet retraction = suction	Stylet retraction

as gastric wall recurrence only occurred in patients treated using EUS-FNA (n = 2), clinicians must consider the potential risks of needle tract seeding, and patients should be carefully selected[74]. Yane *et al*[75] also reported that the 5-year cumulative needle tract seeding rate, estimated using Fine and Gray's method, was 3.8% (95%CI: 1.6%-7.8%). They concluded that, although preoperative EUS-FNA for pancreatic body and tail cancers has no negative effect on recurrence-free survival or overall survival, needle tract seeding after EUS-FNA was observed to have a non-negligible rate[75]. Sakamoto reported that among the six cases with needle tract seeding occurred, it was revealed by upper gastrointestinal endoscopy in four. They suggested upper gastrointestinal endoscopy follow-up, as well as computed tomography, in patients who undergo gastric EUS–FNA before distal pancreatectomy to identify needle tract seeding at soon as possible[76].

CONCLUSION

Since pancreatic cancer treatment is highly invasive and EUS-TA does not worsen cancer-specific prognosis, histological diagnosis should be made to establish effective treatment options. Furthermore, with genomic profiling, a large volume of tissue are needed from each biopsy; Franseen and Fork-tip needles should be the first choice for this purpose. EUS-TA involving three or more punctures using the stylet retraction method and the torque or fanning technique with a 22-G FNB or thicker needle is the most effective tissue-sampling method for patients with solid pancreatic tumors scheduled for treatment, including those receiving personalized medicine. The prediction of MOSE histological specimen volume by measuring MVC length may have clinical significance, especially when submitting specimens for NGS. The application of precision medicine for treating pancreatic ductal adenocarcinoma has just begun, and further development of genetic testing equipment, drugs, tissue collection devices, and puncture technique is required.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Antihepatoma peptide, scolopentide, derived from the centipede scolopendra subspinipes mutilans

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Abstract

BACKGROUND

Centipedes have been used to treat tumors for hundreds of years in China. However, current studies focus on antimicrobial and anticoagulation agents rather than tumors. The molecular identities of antihepatoma bioactive components in centipedes have not yet been extensively investigated. It is a challenge to isolate and characterize the effective components of centipedes due to limited peptide purification technologies for animal-derived medicines.

AIM

To purify, characterize, and synthesize the bioactive components with the strongest antihepatoma activity from centipedes and determine the antihepatoma mechanism.

METHODS

An antihepatoma peptide (scolopentide) was isolated and identified from the centipede *scolopendra subspinipes mutilans* using a combination of enzymatic hydrolysis, a Sephadex G-25 column, and two steps of high-performance liquid chromatography (HPLC). Additionally, the CCK8 assay was used to select the extracted fraction with the strongest antihepatoma activity. The molecular weight of the extracted scolopentide was characterized by quadrupole time of flight mass spectrometry (QTOF MS), and the sequence was matched by using the Mascot search engine. Based on the sequence and molecular weight, scolopentide was synthesized using solid-phase peptide synthesis methods. The synthetic scolopentide was confirmed by MS and HPLC. The antineoplastic effect of extracted scolopentide was confirmed by CCK8 assay and morphological changes again *in vitro*. The antihepatoma effect of synthetic scolopentide was assessed by the CCK8 assay and Hoechst staining *in vitro* and tumor volume and tumor weight *in vivo*. In the tumor xenograft experiments, qualified model mice (male 5-week-old BALB/c nude mice) were randomly divided into 2 groups (n = 6): The scolopentide group (0.15 mL/d, *via* intraperitoneal injection of synthetic scolopentide, 500 mg/kg/d) and the vehicle group (0.15 mL/d, *via* intraperitoneal injection of normal saline). The mice were euthanized by cervical dislocation after 14 d of continuous treatment. Mechanistically, flow cytometry was conducted to evaluate the apoptosis rate of HepG2 cells after treatment with extracted scolopentide *in vitro*. A Hoechst staining assay was also used to observe apoptosis in HepG2 cells after treatment with synthetic scolopentide *in vitro*. CCK8 assays and morphological changes were used to compare the cytotoxicity of synthetic scolopentide to liver cancer cells and normal liver cells *in vitro*. Molecular docking was performed to clarify whether scolopentide tightly bound to death receptor 4 (DR4) and DR5. qRT-PCR was used to measure the mRNA expression of DR4, DR5, fas-associated death domain protein (FADD), Caspase-8, Caspase-3, cytochrome c (Cyto-C), B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), x-chromosome linked inhibitor-of-apoptosis protein and Cellular fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein in hepatocarcinoma subcutaneous xenograft tumors from mice. Western blot assays were used to measure the protein expression of DR4, DR5, FADD, Caspase-8, Caspase-3, and Cyto-C in the tumor tissues. The reactive oxygen species (ROS) of tumor tissues were tested.

RESULTS

In the process of purification, characterization and synthesis of scolopentide, the optimal enzymatic hydrolysis conditions (extract ratio: 5.86%, IC_{50}: 0.310 mg/mL) were as follows: Trypsin at 0.1 g (300 U/g, centipede-trypsin ratio of 20:1), enzymolysis temperature of 46 °C, and enzymolysis time of 4 h, which was superior to freeze-thawing with liquid nitrogen (IC_{50}: 3.07 mg/mL). A peptide with the strongest antihepatoma activity (scolopentide) was further purified through a Sephadex G-25 column (obtained A2) and two steps of HPLC (obtained B5 and C3). The molecular weight of the extracted scolopentide was 1018.997 Da, and the peptide sequence was RAQNHYCK, as characterized by QTOF MS and Mascot. Scolopentide was synthesized *in vitro* with a qualified molecular weight (1018.8 Da) and purity (98.014%), which was characterized by MS and HPLC. Extracted scolopentide still had an antineoplastic effect *in vitro*, which inhibited the proliferation of Eca-109 (IC_{50}: 76.27 μg/mL), HepG2 (IC_{50}: 22.06 μg/mL), and A549 (IC_{50}: 35.13 μg/mL) cells, especially HepG2 cells. Synthetic scolopentide inhibited the proliferation of HepG2 cells (treated 6, 12, and 24 h) in a concentration-dependent manner *in vitro,* and the inhibitory effects were the strongest at 12 h (IC_{50}: 208.11 μg/mL). Synthetic scolopentide also inhibited the tumor volume (Vehicle *vs* Scolopentide, P = 0.0003) and weight (Vehicle *vs* Scolopentide, P = 0.0022) in the tumor xenograft experiment. Mechanistically, flow cytometry suggested that the apoptosis ratios of HepG2 cells after treatment with extracted scolopentide were 5.01% (0 μg/mL), 12.13% (10 μg/mL), 16.52% (20 μg/mL), and 23.20% (40 μg/mL). Hoechst staining revealed apoptosis in HepG2 cells after treatment with synthetic scolopentide *in vitro*. The CCK8 assay and

morphological changes indicated that synthetic scolopentide was cytotoxic and was significantly stronger in HepG2 cells than in L02 cells. Molecular docking suggested that scolopentide tightly bound to DR4 and DR5, and the binding free energies were-10.4 kcal/mol and-7.1 kcal/mol, respectively. In subcutaneous xenograft tumors from mice, quantitative real-time polymerase chain reaction and western blotting suggested that scolopentide activated DR4 and DR5 and induced apoptosis in SMMC-7721 Liver cancer cells by promoting the expression of FADD, caspase-8 and caspase-3 through a mitochondria-independent pathway.

CONCLUSION

Scolopentide, an antihepatoma peptide purified from centipedes, may inspire new antihepatoma agents. Scolopentide activates DR4 and DR5 and induces apoptosis in liver cancer cells through a mitochondria-independent pathway.

Key Words: Scolopendra; Centipede; Antihepatom peptide; Hepatocellular carcinoma; Death receptor 4; Death receptor 5

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Core Tip: Centipedes have been used to treat tumors for hundreds of years in China. However, the molecular identities of their bioactive components have not yet been extensively investigated. We have modified the centipede extraction method and found a polypeptide (scolopentide) with the strongest antihepatoma activity. Mechanistically, scolopentide activated death receptor 4 and death receptor 5 to induce apoptosis through a mitochondria-independent pathway. Scolopentide may be an inspiration for the development of antihepatoma agents. We also aim to provide a reference for the purification and characterization of effective components in animal-derived medicines.

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INTRODUCTION

Centipedes have been widely used in traditional Chinese medicine to treat many diseases, including convulsions, strokes, rheumatic joint diseases, snake bites and tumors[1]. It is a major challenge to isolate and characterize the effective components of centipedes due to limited peptide purification technologies for animal-derived medicines. Currently, the main extraction technologies are as follows: Aqueous extraction[2], organic solvent extraction[2-5], enzymatic hydrolysis[6], ultrasonic extraction[2], and freeze-thaw extraction[2]. To obtain low-weight peptides, extracted proteins require further purification. At present, the main purification technologies of animal proteins are as follows: Sephadex column gel chromatography (GC)[3,7,8], HPLC/RP-HPLC[3,5-8], ion exchange chromatography[7,9], and graded ultrafiltration[7]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a traditional separation and identification technique[10], and mass spectrometry (MS) is the most widely used technique for identifying peptides for use in animal-derived medicines[5-7]. To date, there is no single method that can extract a single peptide from complex mixtures (Table 1). Compound extraction plans are mostly used, which are costly and complicated. Consequently, animal-derived medicines have not been developed as herbal medicines. Currently, the main extracts are still crude peptides, and they have some disadvantages, such as a low extraction ratio, undefined composition, unstable properties and susceptibility[11,12]. These disadvantages may be prevented by identifying and characterizing the effective compositions of animal-derived medicines and synthesizing them *in vitro*.

Some ancient Chinese medical studies have reported that centipedes can be used to treat tumors. "Compendium of Materia Medica"[13], a famous ancient Chinese pharmaceutical work, recorded that centipedes can be used to treat tumors in the chest and abdomen. It is also recorded in the modern Chinese medical literature "Integrating Chinese and Western Medicine"[14] that centipede liquor can be used to treat esophageal cancer with excellent clinical effects. Moreover, the Shendan Sanjie capsule, a medicine containing centipedes, has already been applied to treat cancers and has been proven to inhibit colitis-associated cancer[15]. Although the antitumor theory and clinical efficacy of centipedes have been verified for hundreds of years in China, current experimental studies still focus on antimi-

Table 1 Small molecular extracts of centipede

Name	Extract methods	Function	Ref.
Scolopendrin I	Cation-exchange chromatography and two steps RP-HPLC	Antimicrobial (G^+, G^-, and fungi)	[53]
Scolopin 1	(1) Sephadex Gel Chromatography; (2) RP-HPLC; and (3) characterized by MALDI-TOF MS	Antimicrobial (G^+, G^-, fungi, drug-resistant bacteria)	[37]
Scolopin 2, Scolopin 2-NH2	(1) Sephadex Gel Chromatography; (2) RP-HPLC; and (3) Scolopin2-NH2 was amidation modification from scolopin 2	(1) Antimicrobial (G^+, G^-, fungi, and drug-resistant bacteria); and (2) inhibit proliferation of cervical cancer HeLa cells	[16, 37,45]
LBLP	Transcriptomic data analysis, peptide synthesis	Anti-fungi	[54]
Scolopendrasin II, V, III, X	Next-generation sequencing	(1) Antimicrobial (G^+, G^-, drug-resistant bacteria, fungi); and (2) regulating immune system to antimicrobial indirectly	[40, 55-57]
Scolopendrasin IX	Next-generation sequencing	(1) Regulating immune system to antimicrobial indirectly; and (2) treating autoimmune arthritis	[58]
Scolopendrasin VII	Next-generation sequencing	(1) Antimicrobial (G^+, G^-, drug-resistant bacteria, and fungi); (2) regulating immune system to antimicrobial indirectly; and (3) inhibiting leukemia U937 and jurkat cells	[17, 59,60]
Scolopendin, 1, 2	RNA sequencing	Antimicrobial (G^+, G^-, drug-resistant bacteria, and fungi)	[41-44]
SerGln-Leu (SQL)	(1) Ultrafiltration, Sephadex G-50 column, Source 15Q anion exchange Column; (2) RP-HPLC C18 column; and (3) characterized by ESI-MS	Anticoagulation and antithrombotic	[7]
Thr-Asn-Gly-Tyr-Thr, (TNGYT)	(1) Sephadex G-50 column; (2) RP-HPLC C18 column; and (3) Characterized by ESI-MS	Anticoagulation and antithrombotic	[38]
SsTx-R12A	Red-headed centipede's venom cDNA arsenal	Treating autoimmune diseases mediated by T cells	[61]
SsmTX-I	Red-headed centipede's venom cDNA arsenal	Analgesic	[62]
SsTx	Sephadex G-50 Gel Chromatography and RP-HPLC	Blocking KCNQ potassium channels leads to cardiovascular, respiratory, muscular and nervous system damage	[8]
Ssm6a	Sephadex Gel Chromatography and RP-HPLC	Analgesic	[39]
Haemolymph and tissue extracts	(1) Enzymatic hydrolysis; (2) HPLC; and (3) characterized by MS	Antimicrobial (G^+, G^-, fungi, viruses, and parasites)	[6]
Lacrain	RP-HPLC and characterized by MS	Antibacterial (G^-)	[63]
Pinipesin	(1) Organic solvent extraction; (2) HPLC Jupiter® C18 column; and (3) characterized by MS	Antimicrobial	[5]
AECS	Organic solvent extraction (ethanol)	Inducing apoptosis of human epidermal carcinoma A431 cells and human melanoma HEK293/EGFR, A375 cells	[4,18]
Two isoquinoline alkaloids	(1) Organic solvent extraction (ethanol); (2) sephadex G-25 Gel chromatography; and (3) HPLC	Inducing apoptosis of brain glioma U87 cells	[3]
Centipede water extract, alcohol extract	(1) Water extraction; (2) freeze-thawing with liquid nitrogen, ultrasonic extraction; and (3) organic solvent extraction (ethanol)	No tumour inhibition in S180 and H22 Transplanted tumour mice	[2]
CE, CA	Organic solvent extraction	Inhibiting cervical tumours *in vivo*	[64]
Centipede chloroform extract	Organic solvent extraction (hexane, chloroform, ethanol)	Reducing central nervous system symptoms of Alzheimer's disease	[65]
Scom 5	(1) Superdex 75 gel filtration; (2) RESOURCE S ion chromatography; and (3) characterized by LC-MSMS, MALDI-TOF/TOF and protein sequencing techniques	Human allergens	[11]

HPLC: High performance liquid chromatography; RP-HPLC: Reversed-phase high-performance liquid chromatography; MS: Mass spectrum.

crobial and anticoagulation agents. Only a few studies have observed the antitumor effects of centipedes (Table 1). The potential mechanism is also poorly understood. The tumor types focused on have been gliomas[3], cervical cancer[16], leukemia[17], epidermal cancer[4], and melanoma[18], rather than tumors in the chest and abdomen. Purified centipede peptides can suppress tumor cells by inducing caspase-related apoptosis through a mitochondria-dependent pathway[3,4,16,18], inducing cell cycle arrest[3,18] and necrosis through a specific interaction with phosphatidylserine (Table 1)[17]. Additionally, our previous studies showed that crude centipede peptides could induce apoptosis in

HepG2 cells and lung cancer A549 cells by arresting cells at the G2/M phase[19,20].

Apoptosis has been widely used as a target in cancer treatment[21]. There are two main apoptosis signaling pathways: Mitochondria-dependent and mitochondria-independent pathways. The mitochondria-dependent pathway is controlled by Bcl-2 family proteins and is activated by DNA damage caused by chemotherapy and radiotherapy through activation of the tumor suppressor gene p53[22]. However, the loss of p53 is common in the clinic, contributing to the resistance of conventional chemotherapy and the development of most cancers[23,24]. Thus, the mitochondria-independent pathway, which is independent of p53, has become a promising target[25]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is part of the mitochondria-independent pathway. TRAIL can selectively initiate apoptosis of cancer cells but has no obvious toxic effect on normal cells[26-28], which makes it a promising chemotherapy drug for many cancers. Nonetheless, recent studies have shown that some cancers, including liver cancer and esophageal cancer, are resistant to TRAIL-mediated apoptosis, which greatly reduces its clinical efficacy[29,30]. Current research focuses on finding suitable TRAIL sensitizers and developing TRAIL receptor agonists to maximize the cell-killing effect. However, current studies mainly focus on sensitizers rather than receptor agonists, and the clinical and laboratory results are not ideal[26,28]. Therefore, for research and clinical applications, it would be useful to find stable and efficient TRAIL receptor agonists. Recently, the regulatory effect of Chinese herbal extracts on the TRAIL apoptosis pathway was gradually discovered. Chinese herbal extracts such as luteolin and artemisinin derivatives can induce apoptosis of liver cancer cells by upregulating the expression of death receptor 4 (DR4) or DR5, which are TRAIL receptors on the cell surface[31,32].

In this study, we isolated a novel low-molecular-weight antihepatoma peptide, designated scolopentide, from *Scolopendra subspinipes mutilans L. Koch* and synthesized it *in vitro*. We observed that synthetic scolopentide inhibited tumor proliferation and was more toxic to HepG2 human liver cancer cells than to L02 human embryonic hepatocytes. Mechanistically, scolopentide activated TRAIL receptors (DR4 and DR5), upregulated the expression of fas-associated death domain protein (FADD), caspase-8 and caspase-3 through a mitochondria-independent pathway and finally induced tumor cell apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific Inc., United States) and HyClone (Logan, United States). Fetal bovine serum (FBS) was purchased from Gibco and Invitrogen (Thermo Fisher Scientific Inc., United States). DMSO was purchased from Amresco (Solon, United States). PBS was purchased from Boster (Wuhan, Hubei Province, China) and WellBio (Shanghai, China). Trypsin was purchased from Invitrogen. Penicillin-streptomycin was purchased from Beyotime (Shanghai, China). Hoechst was purchased from SolarBio (Beijing, China).

Cell culture

The Bel-7402 Liver cancer cell line, Eca-109 esophageal cancer cell line and A549 Lung cancer cell line were purchased from the Cell Resource Center of Shanghai University of Biology. The HepG2 Liver cancer cell line and L02 human embryonic hepatocyte cell line were purchased from The Cell Center of Xiangya Medical College, Central South University. The SMMC-7721 liver cancer cell line was purchased from the BeNa Culture Collection (Suzhou, Jiangsu Province, China). Cells were placed in culture flasks (Corning Inc., United States) and cultured in a constant temperature incubator (SHEL-LAB, Hitachi, United States) at 37 °C and 5% CO_2 with saturated humidity. The medium comprised 10% FBS, low-glucose DMEM and penicillin-streptomycin (100 U/mL). Cells were observed under an inverted microscope (Olympus, Japan).

Cell viability assay

The viability of HepG2, A549, Eca-109 and Bel7402 cells treated with different centipede extracts was determined by a Cell Counting Kit-8 (Dojindo, Japan). The viability of HepG2 and L02 cells treated with synthetic scolopentide was also determined. Groups and treatments were as follows: (1) Vehicle group: 100 μL cell suspension + 100 μL complete medium; (2) zero group: 200 μL of complete medium; and (3) scolopentide group: Cells were treated with different centipede extracts/synthetic scolopentide. Three replicate wells were used for each group.

Cells at the logarithmic growth stage were collected, and the density was adjusted to 1×10^4 cells/mL. The cell suspension (100 μL) was plated into a 96-well plate (Corning Inc., United States). Four hours later, drugs were added after the cells had adhered to the well. Twelve hours later, 100 μL culture solution containing 10% CCK8 was added to each well. Cells were incubated for 2 h at 37 °C with 5% CO_2. Then, the absorbance (OD) at 450 nm was analyzed by a microplate reader, and the mean was calculated. Cell viability (%) = scolopentide group OD/vehicle group OD × 100%. The cell suppression ratio (IR) was determined with the following formula: IR (%) = (vehicle group OD-scolopentide group OD)/vehicle group OD × 100%. The zero group OD was subtracted from the OD of all groups for

correction. The 50% maximum inhibitory concentration (IC_{50}) was determined by constructing dose-response curves.

Extraction of Crude Peptides from Scolopendra subspinipes mutilans

Crude centipede peptides were obtained by enzymolysis combined with acetone precipitation. The "Pharmacopoeia of the People's Republic of China (2020)" references the use of the whole dried body of *Scolopendra subspinipes mutilans*[1]. Centipedes were purchased from the pharmacy department of the First Affiliated Hospital of Hunan University of Chinese Medicine. They were identified as *Scolopendra subspinipes mutilans L. Koch* by Chinese herbal experts. Then, the whole bodies of the centipedes were ground into ultrafine powder by low-temperature ultrafine grinding in the Superfine Powder Engineering Research Center of the Ministry of Education of China. First, 100 g of centipede was dried at 50-60 °C. The superfine powder (particle size 1-75 μm) was crushed by a BFM-6 Bailey superfine powder machine (amplitude 100 Hz, grinding temperature 0-10 °C, grinding time 10-15 min). Microscopic image analysis and laser light scattering were used in combination to assess the particle size, and the particle size of more than 95% of the powder was smaller than 75 μm. Finally, the powder was stored at room temperature until subsequent experiments.

We chose an optimized enzymatic hydrolysis method. The extraction process was as follows: 2 g of centipede superfine powder was added to 10 mL of double distilled water, mixed with trypsin, and incubated in water at 55 °C for 4 h. The trypsin was inactivated in a 99 °C water bath for 10 min. Then, the samples were centrifuged at 3500 r/min for 5 min (low temperature ultracentrifuge, Pharmacia Inc., Sweden), and the supernatant was collected. The samples were centrifuged at 5000 r/min at 4 °C for 10 min. The supernatant was collected again. Two volumes of precooled acetone were added to precipitate the former supernatant, which was centrifuged at 5000 r/min at 4 °C for 10 min. The precipitate was dissolved in double steaming water, and the process was repeated twice. Finally, 2 volumes of precooled acetone and petroleum ether (1:1) were added to precipitate again, which was centrifuged at 5000 r/min at 4 °C for 10 min, and the precipitate was collected. Finally, the precipitate was freeze-dried (FD-1 Freeze Dryer, Rikakika Co., Ltd., Tokyo, Japan), weighed, and stored at -20 °C, and the protein content was determined for subsequent experiments.

Optimization of enzymolysis extraction methods

In the process of preparing crude centipede peptides, we tested the influence of single factors on the extraction rate of the crude extract. The five factors included protease type (trypsin, pepsin), water-centipede ratio, protease dose, enzymolysis temperature and enzymolysis time. Then, an $L_9(3^4)$ orthogonal design was adopted to investigate the effects of enzymolysis time, enzymolysis temperature and trypsin dose on crude extracts, which were the three most influential factors in single-factor experiments (Table 2). In addition to the extraction ratio, a CCK8 assay was used to detect the suppression effect of different crude extracts on HepG2 cell viability to determine the optimal technological conditions. We also compared the cytotoxicity of the crude extracts obtained from two methods, freeze-thawing with liquid nitrogen and enzymolysis with acetone precipitation, under optimal technological conditions to determine the significance of this improvement.

Purification and identification of an antihepatoma peptide from crude centipede peptides

Gel chromatography: For the first step, a Sephadex G-25 column was used to isolate the component with the strongest antitumor effect. With 0.1% trifluoroacetic acid-acetonitrile as the mobile phase, gradient elution was performed using an AKTA protein purification system (Amersham Bioscience, United States). In detail, a crude centipede peptide solution (50 mg/mL) was slowly added to a Sephadex G-25 column. Chromatographic columns were eluted with 0.1% trifluoroacetic acid-acetonitrile (flow rate 0.5 mL/min). The resulting columns (3 mL/tube) were collected by an automatic collector. After isolation, tubes with the same peak are collected together, according to the peaks displayed on the computer ultraviolet spectrum. The protein contents of different peaks were determined by a fluorescence microplate reader (TECAN, Austria). The CCK8 assay was used to assess the antitumor activity of different peaks and to select the strongest peak for subsequent experiments.

High-performance liquid chromatography: For further purification, high-performance liquid chromatography (HPLC) was used as the next step. Initially, we used a conventional C18 column, but the results showed that the sample was hydrophilic. Thus, a hydrophilic XAmide column (5 μm, 100 A, 4.6 mm × 250.0 mm) was chosen. The sample solution (1 mg/mL) was filtered through a 0.45 μm microporous membrane and eluted with acetonitrile-water-100 mmol/L ammonium formate (pH 3.5) (flow rate 4 mL/min, detection wavelength 260 mm, injection volume 20 μL). Different fractions were collected according to the HPLC chromatogram peaks. The CCK8 assay was used to select the fraction with the strongest antitumor activity. Then, the above HPLC and CCK8 assays were repeated. The final centipede peptide was concentrated, purified, freeze dried and designated scolopentide.

Determination of the molecular weight and peptide sequence of scolopentide

Quadrupole time of flight mass spectrometry (UPLC-QTOF MS 6530, SHIMADZU, Japan) was used to

Table 2 $L_9(3^4)$ levels of orthogonal experimental design

Level	Trypsin (g)	Time (h)	Temperature (°C)
1	0.1	3	37
2	0.2	4	46
3	0.3	5	55

measure molecular weight. The ion source parameters were as follows: Electrospray ion source, positive ion mode, temperature 300 °C, gas flow 11 L/min, and spray pressure 15 psi. Scolopentide did not pass through the liquid column but went directly through the mobile phase to the QTOF system. The Mascot search engine from Matrix Science was used to match peptide sequences. Based on the protein sequence library of Mascot, the molecular weight of scolopentide was compared with that of known centipede proteins. The amino acid sequence that best matched the molecular weight was selected.

Solid-phase peptide synthesis

Scolopentides that were characterized by QTOF MS and Mascot were synthesized using solid-phase peptide synthesis methods. Peptide synthesis was performed by Genscript (Nanjing, China) as follows: (1) A total of 400 mg chloromethyl resin was selected to synthesize the polypeptides, 20% piperidine/N, n-dimethylformamide was added to the reactor, and the reactor was shaken for 20 min; (2) the solvent was removed by filtration, N, n-dimethylformamide was added to the system, and the reactor was shaken for 1 min, followed by filtering to remove the liquid, which was carried out three times; (3) a total of 150 μl ninhydrin and a small amount of resin were added to the detection tube, which was placed at 100 °C for 20 s, after which the resin's color was checked for change, which indicated that Fmoc was removed successfully; (4) the prepared amino acid solution was added to the reactor, 1 mL of N,N'-diisopropyl carbon diimine/1-hydroxybenzotriazole was added, and the reactor was shaken for 1 h; (5) step 3 was repeated, and if the color did not change, the coupling was considered successful; (6) step 2 was repeated to wash the resin; and (7) the above operation was repeated, and the corresponding amino acids were added until polypeptide synthesis was completed. Then, HPLC was used to test the purity of the synthetic scolopentide, and MS was used to test the molecular weight. Finally, the peptides were freeze-dried and stored at -20 °C until they were used.

Flow cytometry

The apoptosis of HepG2 cells treated with extracted scolopentide was determined by Annexin V-Alexa Fluor660/7-AAD (BB20121, BestBio, China). The cells were digested and collected by trypsin. Approximately 5×10^5 cells were collected after washing with PBS twice and centrifuged at 2000 rpm for 5 min. Then, 100 μL of Annexin V binding buffer was added to resuspend the cells, and the concentration was adjusted to 3×10^6 cells/mL. Five microliters of annexin V-FITC and 5 μL of propidium iodide were added to the cell suspension, mixed and incubated for 10 min at 4 °C in the dark. Then, 400 μL of PBS was added to the cell suspension and mixed. Within 1 h, flow CytoFlex (A00-1-1102, Beckman, United States) was utilized to detect cell apoptosis.

Molecular docking

To investigate the antihepatoma mechanism of scolopentide, Discovery Studio 2020 Client and AutoDock-Vina 1.1.2 were used to predict the binding affinity between scolopentide and DR4 and DR5. BIOVIA Discovery Studio 2020 was applied to process and visualize the results. To obtain molecular structure files, scolopentide structures were constructed with Discovery Studio (Small Molecules) according to the sequence RAQNHYCK. Molecular structure files of DR4 (PDB code 5CIR) and DR5 (PDB code 419X) were retrieved from the RCSB PDB database (https://www.rcsb.org/). The DR4 and DR5 structures were modified by Discovery Studio for water deletion, binding pocket prediction, hydrogenation, and conformation optimization. Then, AutoDock-Vina was used to perform molecular docking and calculate the affinity potential energy of the protein molecule potential binding modes between scolopentide and DR4 and DR5.

Hoechst staining assay

HepG2 cells were inoculated into 6-well plates (1×10^6 cells per well), and 100 μg/mL synthetic scolopentide was added. The cell slides were removed and washed with PBS 2 or 3 times after incubation at 37 °C and 5% CO_2 with saturated humidity for different durations (6 h, 12 h, and 24 h). Then, the slides were fixed with 4% paraformaldehyde for 30 min and rinsed with PBS 3 times. Hoechst (working concentration: 25 μM) was added to the slides, which were incubated for 3 min and rinsed with PBS 3 times. Finally, the slides were sealed with glycerin buffer, stored away from light and observed under a fluorescence microscope (Zeiss, Germany).

Animal and tumor xenograft experiments

Male 5-wk-old BALB/c nude mice (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were chosen for the formation of subcutaneous xenograft tumors. SMMC-7721 cells (0.2 mL/mouse, 1×10^7 cells/mL) were subcutaneously injected into the right back. The tumor diameter reached 1 cm at 3 wk. Qualified model mice were randomly divided into 2 groups (n = 6 per group): The scolopentide group (intraperitoneal injection of synthetic scolopentide, 500 mg/kg/d) and the vehicle group (same volume of normal saline). The length and width of tumors were measured every 2 d, and volume was calculated as the maximum tumor length × width^2 × 0.5. After 14 d of treatment, all mice were euthanized by cervical dislocation, and tumor weights were measured. All operations followed the United Kingdom Animals (Scientific Procedures) Act, 1986. All laboratory animals were carefully handled, and the study was reviewed and approved by the Ethical Review Committee of Experimental Animal Welfare at Hunan University of Chinese Medicine (Approval No. LL2021040705).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from tumor tissues based on the instructions of the RNA Simple Total RNA Kit (Novoprotein Scientific Inc., Suzhou, Jiangsu Province, China), and the RNA concentration was measured by a microplate reader. Then, NovoScript®Plus (Novoprotein Scientific Inc., Suzhou, China) was utilized to reverse-transcribe the RNA into cDNA, which was stored at -20 °C until subsequent use. According to the instructions of NovoStart®SYBR (Novoprotein Scientific Inc., Suzhou, Jiangsu Province, China), 10 μL SuperMix Plus, 7 μL RNase Freewater, 2 μL cDNA, 0.5 μL forward primers and 0.5 reverse primers (Genecreate Inc., Wuhan, Hubei Province, China) were mixed. GAPDH was used as the internal reference. A LightCycler®96 (Roche, Switzerland) was used for quantitative real-time polymerase chain reaction (qRT-PCR). The primer sequences are shown in Table 3. The qRT-PCR conditions were as follows: Predenaturation for 60 s at 95 °C; 45 cycles of denaturation for 20 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C; 1 cycle of melting for 10 s at 95 °C, 60 s at 65 °C, and 1 s at 97 °C; and cooling for 30 s at 37 °C. Data were individually normalized to the mean of the relative expression of GAPDH. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. The experiment was repeated at least three times, and the results that were the most representative are shown.

Western blotting

Tumor tissue specimens were prepared and suspended in ice-cold RIPA lysis buffer (CWBIO Co., Ltd, Beijing, China) and incubated for 20 min. The supernatant was removed after centrifugation. The protein levels of tumor tissues were determined by the BCA assay using a BCA Protein Assay Kit (CWBIO Co., Ltd, Beijing, China). Proteins (50 μg) were mixed with pure water and SDS loading buffer and denatured at 100 °C for 10 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Merck Millipore Ltd., Germany). Then, PVDF membranes were blocked for 1 h in blocking buffer (5% milk in TBST). The membranes were removed and washed with PBST (PBS + 0.1% Tween 20) 3 times, incubated with primary antibodies overnight at 4 °C, washed with TBST 3 times, incubated with Proteintech HRP Goat Anti-Rabbit IgG (H + L) (SA00001-2, diluted 1:6000) for 90 min at room temperature and washed with TBST 3 times. Protein signals were visualized by enhanced chemiluminescence using an eECL western blot kit (CWBIO Co., Ltd, Beijing, China). Membrane images were collected by an Amersham Imager 600 (GE Healthcare, United States) and qualified by Quantity One software. All experiments were repeated three times. Primary antibodies against DR5 (ab199357, diluted 1:1000), FADD (ab108601, diluted 1:1000), cleaved caspase-3 (ab32042, diluted 1:1000), and cytochrome c (Cyto-C) (ab133504, diluted 1:5000) were obtained from Abcam (Cambridge, England). Antibodies against GAPDH (10494-1-AP, diluted 1:5000) and DR4 (24063-1-AP, diluted 1:2000) were obtained from Proteintech Group, Inc. (Chicago, United States). The antibody against cleaved caspase-8 (AF5267, diluted 1:1000) was obtained from Affinity Biosciences, Inc. (Jiangsu, China).

Analysis of intracellular reactive oxygen species accumulation

Reactive oxygen species (ROS) were measured using a ROS Assay Kit (E004-1-1 Jiancheng, Nanjing, China) according to the manufacturer's instructions. Briefly, the tumor tissue was prepared into a single cell suspension by enzymatic hydrolysis. Three groups of tubes were set up as follows: (1) Negative vehicle tube: cells without any treatment were resuspended in 0.01 M PBS; (2) positive vehicle tube: cells were resuspended in diluted 2,7-dichlorofluorescin diacetate (DCFH-DA), and an ROS hydrogen donor (concentration: 30 μM) was added to induce the cells; and (3) sample tube: cells were resuspended in diluted DCFH-DA (cell density: 1×10^6-2×10^7/mL). Then, all cells were incubated for 50 min at 37 °C. After labeling with the probe, the single-cell suspensions were subsequently collected and centrifuged for 5-10 min. The precipitates were collected and washed with 0.01 M PBS 1-2 times. The precipitates were centrifuged and collected again. Finally, the cell precipitates were resuspended in PBS and measured on a fluorescence microplate reader (optimum excitation wavelength 500 nm, optimum emission wavelength 525 nm). All results are expressed as fluorescence values.

Table 3 Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Sequence (5' to 3')	
GAPDH	F: AGAAGGCTGGGGCTCATTTG	R: AGGGGCCATCCACAGTCTTC
DR4	F: CCAGGCAGCATTGAAGTCAG	R: GTTTCCAGCATCACCAGGGT
DR5	F: CCCTGGAGTGACATCGAATG	R: CAGCCACAATCAAGACTACGG
FADD	F: TGCGGGAGTCACTGAGAATC	R: GGAGGTAGATGCGTCTGAGTTC
Caspase-8	F: GAGAAGCAGCAGCCTTGAAG	R: GACAGTATCCCCGAGGTTTG
Caspase-3	F: CCCTCCTCAGCATCTTATCC	R: TGGTACAGTCAGAGCCAACCT
Cyto-C	F: CACAAGACTGGGCCAAATCTC	R: CCAGGGATGTACTTCTTGGGA
XIAP	F: CTATGCTCACCTAACCCCAAG	R: TTCTGACCAGGCACGATCAC
c-FLIP	F: GGAACCCTCACCTTGTTTCG	R: CTCCTTGCTTATCTTGCCTCG
Bcl-2	F: CCTTCTTTGAGTTCGGTGGG	R: CGGTTCAGGTACTCAGTCATCC
Bax	F: CACCAGCTCTGAGCAGATCA	R: TGTTACTGTCCAGTTCGTCCC

DR4: Death receptor 4; DR5: Death receptor 5; FADD: Fas-associated death domain protein; Cyto-C: Cytochrome C; XIAP: X-chromosome linked inhibitor-of-apoptosis protein; c-FLIP: Cellular Fas associated death domain-like interleukin-1β converting enzyme inhibitory protein; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein.

Statistical analysis

SPSS 25.0 and GraphPad Prism 8.3 software were used for all statistical analyses. Unless otherwise stated, $P < 0.05$ was considered statistically significant. All data are expressed as the mean ± SD. For the cell viability assay, ANOVA was used to compare the means of multiple groups, Student's *t* test was used for pairwise comparisons, and Fisher's exact test was used for ratio comparisons. For tumor weight, qRT-PCR and WB, Student's *t* test was used when normality and ANOVA were satisfied. For ROS assessment, the Mann-Whitney rank-sum test was used due to unsatisfactory normality. Two-way ANOVA was used for tumor volume analysis.

RESULTS

Crude centipede peptides extracted by optimal enzymolysis hydrolysis extraction ratio of crude peptides under different conditions

Based on the extract ratio and protein content, we carried out five single-factor experiments and finally obtained the optimum conditions: the protease was trypsin, the water-centipede ratio was 5:1, the protease dose was 0.1 g (300 U/g, with a centipede-trypsin ratio of 20:1), the enzymolysis temperature was 46 °C, and the enzymolysis time was 4 h. The three most influential factors were enzymolysis time, enzymolysis temperature and trypsin dose. Therefore, an $L_9(3^4)$ orthogonal design was adopted to further investigate the optimal group of three factors on the extract ratio, as shown in Table 4. In conclusion, Group 4 (A2B1C2), Group 2 (A1B2C2), and Group 9 (A3B3C2) had higher extract ratios.

Cytotoxicity of crude peptides under different conditions

The $L_9(3^4)$ orthogonal design was adopted to further investigate the optimal group of the three factors for cytotoxicity in HepG2 cells, as shown in Table 5. A CCK8 assay was used to evaluate HepG2 cell viability. The results indicated that the 9 extracts induced cell death in a concentration-dependent manner ($P < 0.05$). Extracts from Groups 4, 2, and 9 showed stronger cytotoxicity, and Groups 1, 3, 5, 6, 7, and 8 showed moderate cytotoxicity. In the $L_9(3^4)$ orthogonal experiment, Group 2 (5.86%, 0.310 mg/mL), which used the optimal process of the single-factor experiment, showed little difference from the nonoptimal process of Group 9 (5.94% and 0.278 mg/mL). Indeed, Group 2 required less time and a lower trypsin dose. Based on the IC_{50}, extract ratio and suppression ratio shown in Table 5, Group 2 was ultimately determined to have the optimal extraction process. The optimal extraction conditions were as follows: Trypsin at 0.1 g (300 U/g, centipede-trypsin ratio of 20:1), enzymolysis temperature of 46 °C, and enzymolysis time of 4 h.

Table 4 Extract ratio analysis of $L_9(3^4)$ orthogonal experimental design

Run	A (mg, trypsin)	B (min, time)	C (°C) (temperature)	D (blank)	Extract ratio (%)
1	1	1	1	1	4.55
2	1	2	2	2	5.86
3	1	3	3	3	4.32
4	2	1	2	3	5.63
5	2	2	3	1	4.21
6	2	3	1	2	3.98
7	3	1	3	2	3.34
8	3	2	1	3	3.68
9	3	3	2	1	5.94
K1	14.73	13.52	12.21	14.70	-
K2	13.82	16.01	14.83	13.18	-
K3	12.96	11.64	11.87	13.63	-
R	1.77	4.37	2.96	1.52	-

K*ij* parameters in a first level of the first i-j-average quality index; *I* = 1, 2, 3, representative of 3 levels; *j* = 1, 2, 3; representing three factors.

Table 5 Inhibitory effects of crude centipede peptides on HepG2 cells in different conditions

Concentration (mg/mL)	Suppression ratio (%)								
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
0.039	12.510	6.880	12.070	5.394	3.389	3.213	0.570	1.641	7.094
0.078	22.270	11.240	13.060	16.430	12.890	4.709	2.280	4.540	9.883
0.156	45.770	36.430	45.680	52.290	18.610	16.620	26.110	11.540	33.170
0.3125	85.510	85.040	88.540	94.360	68.110	63.660	58.500	64.170	76.610
0.625	89.090	86.060	92.440	94.520	94.170	94.96	83.200	95.180	94.570
1.250	91.840	87.490	93.050	94.770	96.780	95.730	94.660	95.300	96.000
2.500	92.070	89.470	93.430	94.690	96.940	96.670	95.540	95.670	96.920
5.000	92.750	92.000	95.800	96.680	97.720	97.780	95.880	95.790	97.650
IC_{50} (mg/mL)	0.457	0.310	0.480	0.360	0.584	0.681	0.812	0.757	0.278
Extract ratio (%)	4.550	5.860	4.320	5.630	4.210	3.980	3.340	3.680	5.940

Cytotoxicity of crude peptides from the optimal enzymolysis hydrolysis and freeze-thawing with liquid nitrogen

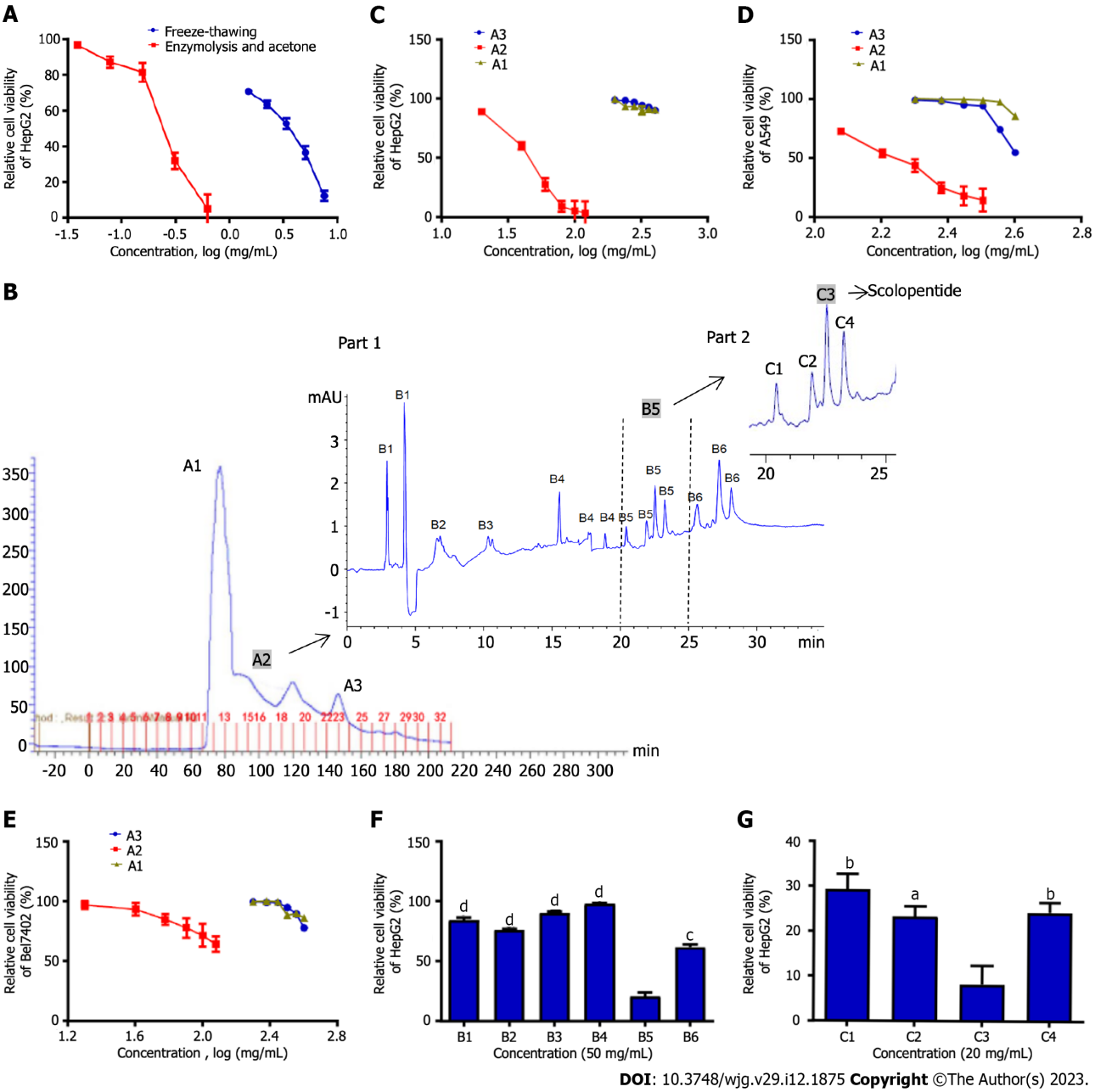
HepG2 cells were treated with crude peptides extracted using two extraction methods. The results showed that extracts obtained from the two methods significantly inhibited HepG2 cells in a concentration-dependent manner. Optimal enzymatic hydrolysis (IC_{50}: 0.31 mg/mL) was superior to freeze-thawing with liquid nitrogen (IC_{50}: 3.07 mg/mL), and the cytotoxicity of extracts increased nearly 10 times (Figure 1A).

Purification of scolopentide from crude centipede peptides

We purified and screened crude centipede peptides three times and obtained a low-molecular-weight peptide with the strongest antihepatoma effect, which was designated scolopentide.

The first purification step (Part A): Separation by a sephadex g-25 column

Sephadex G-25 gel chromatography was used, and the chromatogram obtained showed 3 peaks (Figure 1B). Part A samples (sample ID nos. A1 to A3) were then collected based on 3 peaks and assessed by a microplate reader. The protein and polypeptide contents of A1, A2 and A3 were 55, 40



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Figure 1 Purification of scolopentide from crude centipede peptides. A: The CCK8 assay showed the cytotoxicity of extracts from two methods, and optimal enzymatic hydrolysis was superior to freeze-thawing with liquid nitrogen; B: After the first purification, the Sephadex G-25 chromatogram showed 3 peaks, and A2 was chosen for further isolation; after the second purification, the high-performance liquid chromatography (HPLC) chromatogram showed 6 parts, and B5 was chosen for further purification (part 1); after the third purification, the HPLC chromatogram showed 4 parts, and C3 was chosen for further purification (part 2); C-E: Relative cell viability of HepG2 (C), A549 (D), and Bel7402 (E) cells treated with A1-3 at different concentrations (mg/mL). A2 showed stronger suppression than A1 and A3; F: The CCK8 assay showed that B5 (50 μg/mL) had the strongest suppression of HepG2 cells among B1-6; G: The CCK8 assay showed that C3 (20 μg/mL) had the strongest suppression of HepG2 cells among C1-4. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, and $^{d}P < 0.0001$.

and 20 μg/mL, indicating that samples A1-3 were mainly composed of protein. Since the separation range of the Sephadex G-25 column was 1-5 kDa, we speculated that A1 contained proteins > 5 kDa and that A2 and A3 were composed of low-molecular-weight polypeptides (1-5 kDa). Subsequently, a CCK8 assay was used to detect the suppressive effects of A1, A2 and A3 on HepG2, Bel-7402 and A549 cells (Figure 1C-E). The CCK8 assay showed the following: (1) A2 showed the most significant inhibition of proliferation of the three tumor cell lines among samples A1-3 ($P < 0.05$); (2) A2 showed the strongest inhibitory effect on HepG2 cells: The IC_{50} values of A2 against the three tumor cell lines were 50.1 mg/mL (HepG2), 132.8 mg/mL (Bel-7402) and 154.5 mg/mL (A549); and (3) A1-3 showed an inhibitory effect on the three tumor cell lines in a concentration-dependent manner.

The second purification step (Part B): Purification by HPLC

In Part B, A2, which had the best antineoplastic effect *in vitro* among samples A1-3, was used. HepG2

cells, which were the most strongly inhibited among the three tumor cell lines, were used. First, tricine-SDS-PAGE[10] was performed to further purify this sample in Part B, but the peptide separation was not obvious, and the band patterns were light and diffuse. The reason may be that the molecular weight of the sample was too low for separation by tricine-SDS-PAGE. However, satisfactory results were obtained from HPLC. Therefore, HPLC was adopted for further purification. The chromatogram indicated that the sample from Part B was composed of a variety of peptides with very similar and low molecular weights in 6 peaks (Figure 1B; part 1). Subsequently, the Part B sample was collected in 6 parts (sample ID nos. B1 to B6) based on 6 peaks. A CCK8 assay was used to assess the suppressive effect of B1-6 on HepG2 cells cultured for 48 h, which were the tumor cells that were most sensitive to the centipede extracts. The CCK8 assay showed that B1-4 hardly inhibited the proliferation of HepG2 cells, while B5 and B6 inhibited proliferation ($P < 0.05$), with B5 having the strongest effect (Figure 1F).

The third purification step (Part C): Purification by HPLC

In Part C, the B5 sample, which had the best antihepatoma effect *in vitro* among B1-6, was used. HPLC was adopted for purification, and the chromatogram showed 4 peaks (Figure 1B; part 2). Then, the sample from Part C was collected in 4 parts (C1-4) based on the 4 peaks. The CCK8 assay showed that C3 had the strongest inhibitory effect on HepG2 cells cultured for 48 h, while C1, C2, and C4 showed relatively weak inhibition of proliferation (Figure 1G). Finally, we concentrated and purified C3 and designated it scolopentide.

Characterization of scolopentide

The peptide sequence of the extracted scolopentide is RAQNHYCK, and the mass spectrum (Figure 2A) showed that its molecular weight was 1018.997 Da (the highest peak). Mascot was used to determine the specific peptide sequence of scolopentide, and the final matched sequence was RAQNHYCK, which conformed to the restriction site of the protease. The molecular structure of scolopentide ($C_{42}H_{66}N_{16}O_{12}S$) is shown in Figure 2A (the asterisk).

Synthesis and detection of scolopentide

Scolopentide was synthesized *in vitro* according to the peptide sequence RAQNHYCK, the length of which is 8 AA. Then, MS was used to detect the molecular weight. The observed molecular weight of synthetic scolopentide was 1018.8 Da (Figure 2B), which was similar to that of the extract (1018.997 Da). HPLC was used to assess the purity. The chromatogram is shown in Figure 2C, and the peak proportion is shown in Figure 2D. The synthetic scolopentide corresponds to peak 3; thus, its purity was inferred to be 98.014% according to the area fraction of peak 3. Consequently, the purity and molecular weight of synthetic scolopentide met the requirements.

Antineoplastic effect of extract scolopentide in vitro

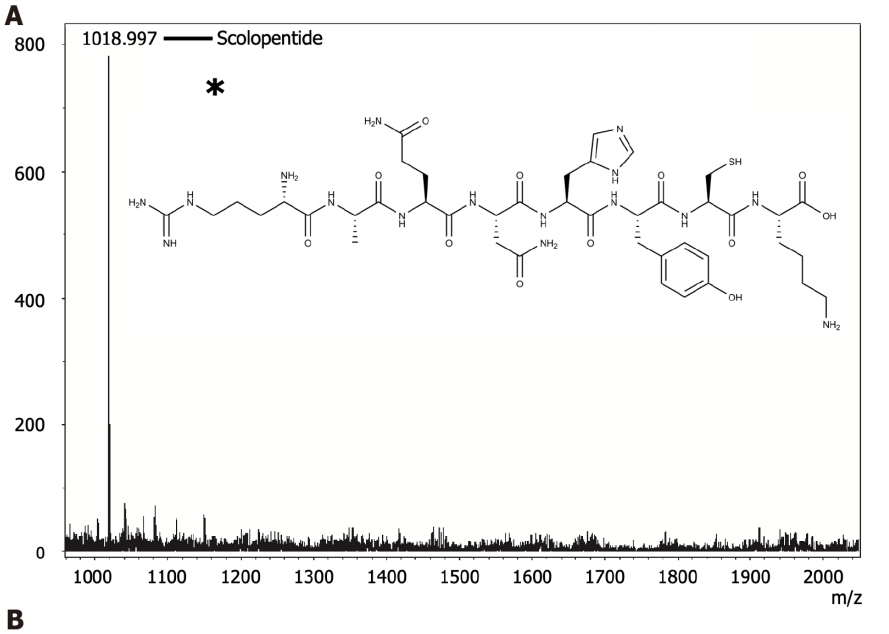
Eca-109, HepG2, and A549 cells were treated with the extracted scolopentide. A CCK8 assay showed that the IC_{50} values in the three cell types were 76.27 μg/mL (Eca-109), 22.06 μg/mL (HepG2), and 35.13 μg/mL (A549) (Figure 3A). Morphological changes in the three cell lines showed that the A549 and HepG2 cells changed considerably (Figure 3B). Based on the CCK8 assay and morphological changes, the following conclusions were drawn: extracted scolopentide still has antineoplastic effects *in vitro*, which inhibited the proliferation of three tumor cell lines, especially HepG2 cells.

Antihepatoma effect of synthetic scolopentide in vitro and in vivo

We wanted to determine whether synthetic scolopentide can exert an antihepatoma effect. Our experiments suggested that synthetic scolopentide had antihepatoma effects *in vitro* and *in vivo*, which may be related to apoptosis induction. *In vitro*, the CCK8 assay suggested that synthetic scolopentide inhibited the proliferation of HepG2 cells in a concentration-dependent manner. The inhibitory effects were the strongest at 12 h, and there was no significant difference between 24 h and 48 h (Figure 3C). Moreover, a Hoechst staining assay was used to observe the morphological changes in HepG2 cells treated with scolopentide (100 μg/mL) (6 h, 12 h, and 24 h). The results showed that cytoplasmic staining and nuclear pyknosis occurred after treatment for 12 h and 24 h, which indicated apoptosis. Apoptosis was most clearly observed at 12 h (IC_{50}: 208.11 μg/mL), consistent with the CCK8 assay (Figure 3D). *In vivo*, the tumor xenograft experiment suggested that the mean tumor volume of the two groups increased gradually, but that of the scolopentide group grew slower. The tumor weight of the scolopentide group was lower than that of the vehicle group (Figure 3E-G).

Cytotoxicity of synthetic scolopentide to L02 cells and HepG2 cells

CCK8 assays and morphological changes were used to compare the cytotoxicity of synthetic scolopentide to liver cancer cells and normal liver cells. After treatment with synthetic scolopentide for 12 h, the CCK8 assay suggested that the cytotoxicity in L02 cells was significantly lower than that in HepG2 cells (100, 150, and 200 μg/mL) (Figure 4A). Morphological changes in the scolopentide group (100 μg/mL) and vehicle group (0 μg/mL) are shown in Figure 4B. Compared to the vehicle group, most HepG2 cells in the scolopentide group died, while some L02 cells survived. The CCK8 assay and



D

Detector A Channel 1 220nm

Peak#	Ret. time	Area	Height	Area%
1	10.373	9024	2400	0.094
2	10.638	22488	5890	0.235
3	10.750	9362274	1583358	98.014
4	10.929	101307	28820	1.061
5	11.115	16570	3086	0.173
6	11.297	40358	8449	0.423
Total		9552020	1632003	100.000

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Figure 2 Characterization of extracted scolopentide and detection of synthetic scolopentide. A: Mass spectrum of extracted scolopentide; the

highest peak indicates the active peptide (scolopentide). The observed molecular weight was 1018.997 Da; the asterisk "∗" means molecular structure of scolopentide; B: Mass spectrum of synthetic scolopentide. The observed molecular weight was 1018.8 Da; C and D: HPLC chromatogram of synthetic scolopentide (C); the highest peak (peak 3) indicates the active peptide, and the area % of peak 3 indicates the purity of synthetic scolopentide (98.014%) (D).

morphological changes indicated that synthetic scolopentide was cytotoxic and was significantly stronger in HepG2 cells than in L02 cells.

Molecular docking of scolopentide with DR4 and DR5

Scolopentide was more cytotoxic in HepG2 cells than in L02 cells (Figure 4), which is similar to the activity of TRAIL, which selectively initiates apoptosis in cancer cells without significant toxicity to normal cells. Among the known TRAIL receptors, only DR4 and DR5 are able to induce apoptosis[33]. In addition, Hoechst staining indicated that HepG2 cells underwent apoptosis after treatment with synthetic scolopentide (Figure 3D). Therefore, we hypothesized that scolopentide could induce apoptosis by activating DR4/DR5. The binding free energies of scolopentide to DR4 and DR5 were-10.4 kcal/mol and-7.1 kcal/mol, respectively. Molecular docking suggested that scolopentide tightly bound to DR4 (Figure 5A) and DR5 (Figure 5B), which supported our hypothesis.

Apoptosis of HepG2 cells treated with extracted scolopentide

Flow cytometry suggested that apoptosis occurred in HepG2 cells after treatment with extracted scolopentide *in vitro*. The apoptosis ratios were 5.01% (0 μg/mL), 12.13% (10 μg/mL), 16.52% (20 μg/mL), and 23.2% (40 μg/mL), which indicates concentration-dependence (Figure 6A).

The antihepatoma mechanism of scolopentide was determined to be induction of apoptosis by activating the TRAIL pathway

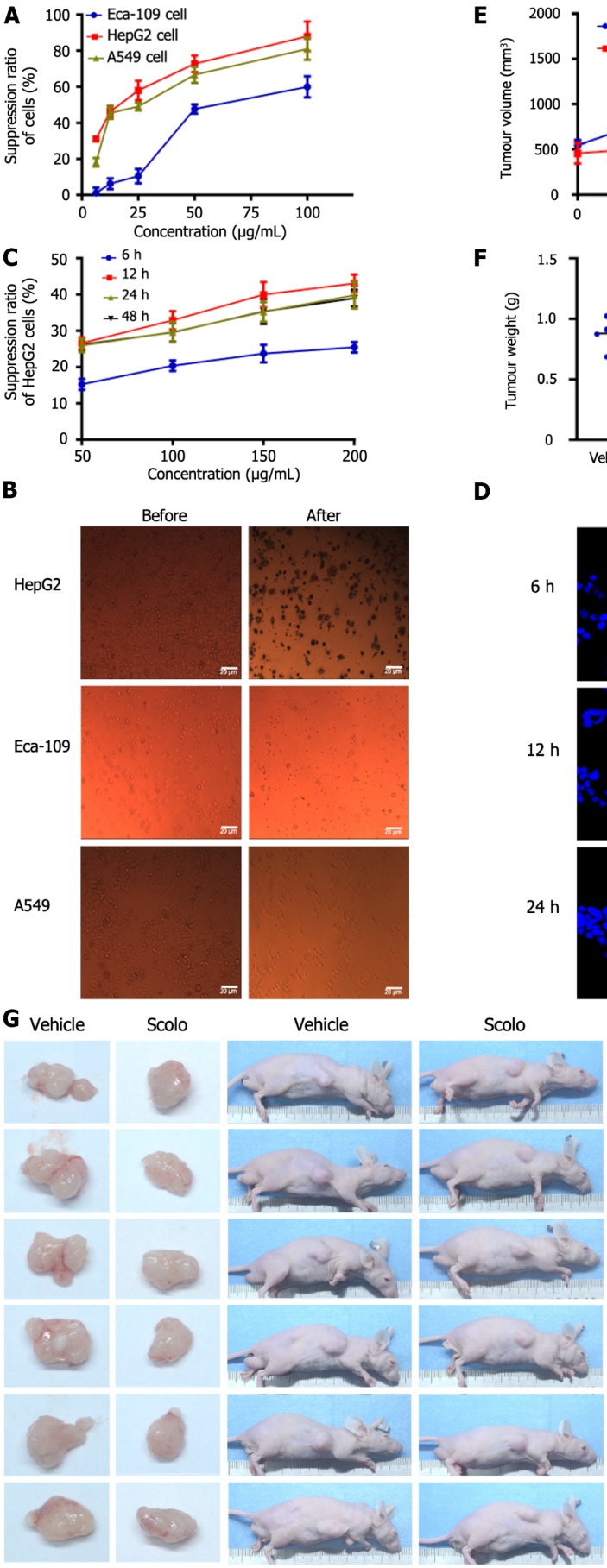
Animal experiments were used to further verify our hypothesis that scolopentide can stimulate the TRAIL pathway to induce apoptosis. The results of qRT-PCR and western blotting suggested that scolopentide activated DR4 (Figure 6B and C) and DR5 (Figure 6H and I), which promoted the expression of FADD (Figure 6E and F), activated the apoptosis promoter caspase-8 (Figure 6F and G) and the executor caspase-3 (Figure 6C and D), and finally induced apoptosis in SMMC-7721 Liver cancer cells. This form of apoptosis appears to be more related to the mitochondria-independent pathway, as Cyto-C and Bcl-2-associated X protein/B-cell lymphoma-2 (Bax/Bcl-2), which are key indicators in the mitochondria-dependent pathway, showed only slight upregulation and insignificant differences (Figure 6I-K). In addition, ROS levels were upregulated (Figure 6L). Cellular fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein (c-FLIP), an inhibitory protein of caspase-8, was downregulated (Figure 6M), and x-chromosome linked inhibitor-of-apoptosis protein (XIAP), an inhibitory protein of caspase-3, was insignificantly upregulated (Figure 6N).

DISCUSSION

Peptides from centipedes have potential antitumor activity that should not be ignored. We isolated and purified crude centipede peptides 3 times and finally obtained a low molecular weight peptide (scolopentide) with the strongest antihepatoma effect. We further demonstrated that mechanistically, scolopentide induced apoptosis in liver cancer cells by activating DR4 and DR5 and promoting the upregulation of FADD, caspase-8 and caspase-3.

Centipedes, an important part of animal Chinese medicine, have played an important role in clinical treatment[1]. The remarkable activities and novel structure also give bioactive components of centipedes enormous potential to be exploited and modified as biological drugs[34]. However, pharmacological studies of centipedes are far behind those of other animals, such as snakes and scorpions[34,35]. Similar to animal-derived medicines, most centipede extracts are still crude. These crude extracts may contain a large number of histamines, polypeptide toxins and other biologically active substances[34], leading to allergic reactions such as itching or serious adverse reactions such as acute myocardial infarction, arrhythmia, tissue necrosis, respiratory depression and hemolysis, which hinder the clinical use of centipedes[11,36]. Small molecular substances from centipedes may not cause adverse reactions and may play a critical role with precise effects.

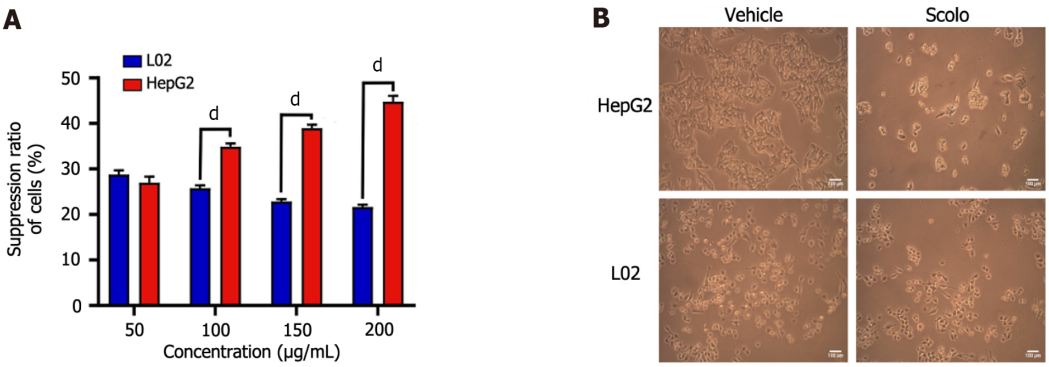
We tried a variety of ways to obtain a higher extraction rate and more accurate activity. For protein extraction, the optimal conditions were determined: 0.1 g trypsin (300 U/g, centipede-trypsin ratio of 20:1), enzymolysis temperature of 46 °C and enzymolysis time of 4 h. For peptide purification, GC[37-39] and HPLC[3,5,6], widely used techniques, were adopted to further purify crude centipede peptides. Mass spectrometry[5-7], the most widely used technique for peptide characterization, was used to identify scolopentide. The object of extraction and method used here are also noteworthy. The whole body of the centipede was chosen for extraction as recorded in Chinese works[14], even though most



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Figure 3 Antihepatoma effect of scolopentide. A: The CCK8 assay showed the suppression ratio of Eca-109, HepG2, and A549 cells treated with extracted

scolopentide at different concentrations; B: Morphological changes in Eca-109, HepG2, and A549 cells under a light microscope (× 40); after treatment with extracted scolopentide, three cells were morphologically changed, especially HepG2 cells; C: The CCK8 assay showed the suppression ratio of HepG2 cells treated with synthetic scolopentide at different times (6 h, 12 h, 24 h, and 48 h) and different concentrations (50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL); D: Hoechst 33342 staining (× 400) of HepG2 cells. After treatment with synthetic scolopentide for 12 h and 24 h, cytoplasmic highlight staining and nuclear pyknosis occurred; E-G: Tumor volume and weight of the scolopentide group (synthetic scolopentide 500 mg/kg/d) and vehicle group (constant volume of normal saline). $n = 6$, $^{b}P < 0.01$, $^{c}P < 0.001$.



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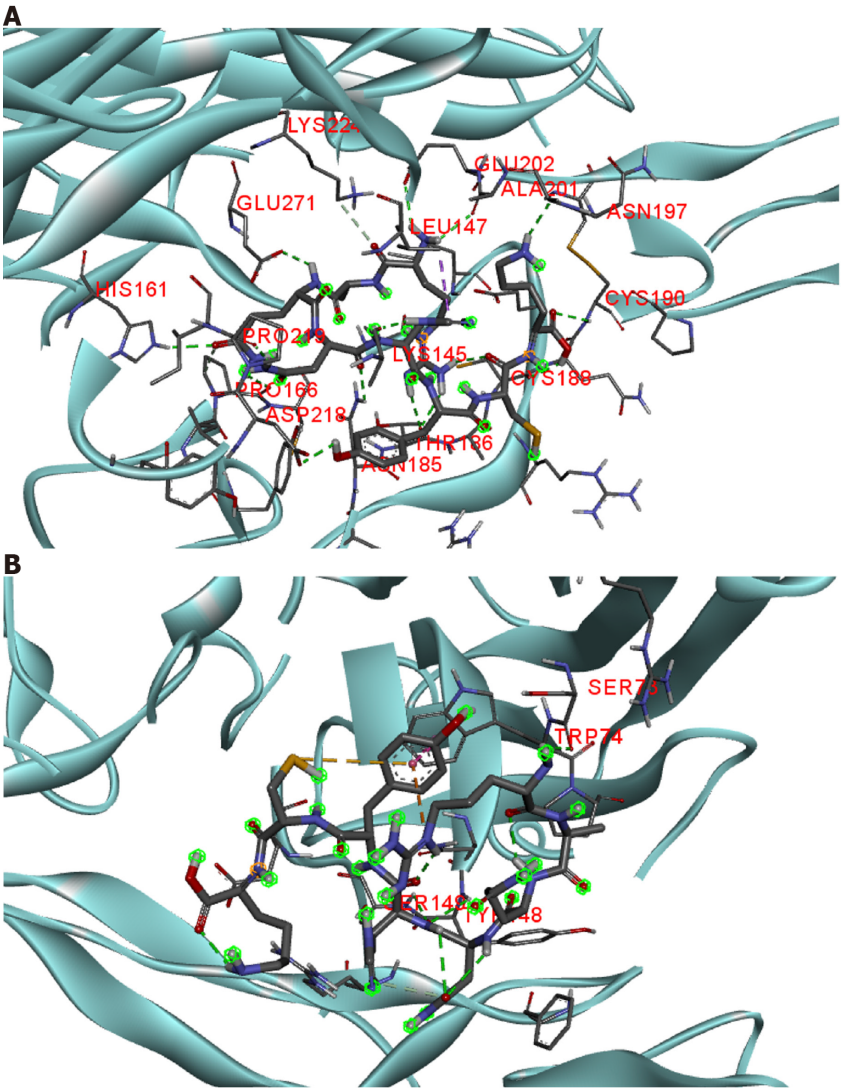
Figure 4 Cytotoxicity of synthetic scolopentide to L02 cells and HepG2 cells. A: The CCK8 assay showed that cytotoxicity to L02 cells was significantly lower than that to HepG2 cells after treatment with synthetic scolopentide for 12 h (100 μg/mL, 150 μg/mL, and 200 μg/mL); B: Morphological changes in HepG2 and L02 cells under a light microscope after treatment with synthetic scolopentide for 12 h (× 100). Compared to cells in the vehicle group (0 μg/mL), most HepG2 cells in the scolopentide group (100 μg/mL) died, while some L02 cells survived. $^{d}P < 0.0001$.

current studies focus on the venom[12,34]. In addition, enzymatic hydrolysis[6], a special extract technique, was chosen for the initial extraction to obtain crude centipede peptides. We aimed to simulate the environment of Chinese medicine being digested by various proteinases in the stomach after oral administration.

Currently, studies on the antihepatoma components of centipedes are rare (Table 1). Thus, we isolated, purified, and screened the crude centipede peptides three times. Finally, we obtained scolopentide (1018.997 Da), which had the strongest antihepatoma activity. Moreover, the peptide sequence RAQNHYCK of scolopentide was matched with the Mascot search engine, which helped to achieve scolopentide synthesis *in vitro*. Mascot also revealed that scolopentide was supposed to be a polypeptide from centipede venom proteins located in Kappa-scoloptoxin (07)-Ssm²a OS. It is worth noting that the tumor types used for screening were tumors in the chest and abdomen, such as liver cancer and esophageal cancer, as recorded in ancient Chinese works[13]. Liver cancer cells were more sensitive than other tumor cells and were chosen for subsequent mechanistic exploration (Figure 3A).

Antimicrobial activity is the most common pharmacological characteristic known among isolated centipede peptides at present (Table 1). Centipede peptides mainly destroy the integrity of cell membranes, which leads to the death of microbes[40-42]. Inducing apoptosis is the second major mechanism by which centipede peptides induce resistance to microorganisms (Table 1). Two antimicrobial centipede peptides, scolopendin and scolopendin 1, were obtained by RNA sequencing[41,43]. They have been shown to cause ROS accumulation in Candida albicans, which leads to mitochondrial depolarization, thus releasing Cyto-C into the cytoplasm from mitochondria and resulting in an increase in Ca^{2+} in the cytoplasm and mitochondria, ultimately inducing caspase-related apoptosis[43,44]. Some centipede peptides can also block the cell cycle and affect the expression of genes related to DNA replication and repair[45]. Additionally, some scholars have shown that a few antimicrobial centipede peptides also have antitumor activity[17,26]. Distinct from the antimicrobial mechanism, the antitumor activities are mainly related to apoptosis, especially the mitochondrial-dependent pathway[3,4,18]. To date, there have been no studies related to the mitochondrial-independent pathway of centipede effects (Table 1).

DR4 and DR5 are involved in the mitochondria-independent apoptosis pathway, which can receive extracellular death signals and induce intracellular apoptosis[46]. FADD receives apoptosis signals transmitted from DR4/DR5 and forms the death-inducing signaling complex TRAIL-DR4/DR5-FADD-procaspase. Then, caspase-8 is activated to initiate two apoptotic pathways: (1) The mitochondria-independent pathway, in which caspase-8 directly activates caspase-3; and (2) the mitochondria-dependent pathway, in which caspase-8 cleaves Bid, resulting in tBID that triggers the release of Cyto-C. Then, Cyto-C successively activates the promoter caspase-9 and the executor caspase-3. ROS accumulation can cause mitochondrial permeability transition pore opening, also leading to Cyto-C release. Apoptosis is the common end of these two pathways (Figure 7)[28,47-49].

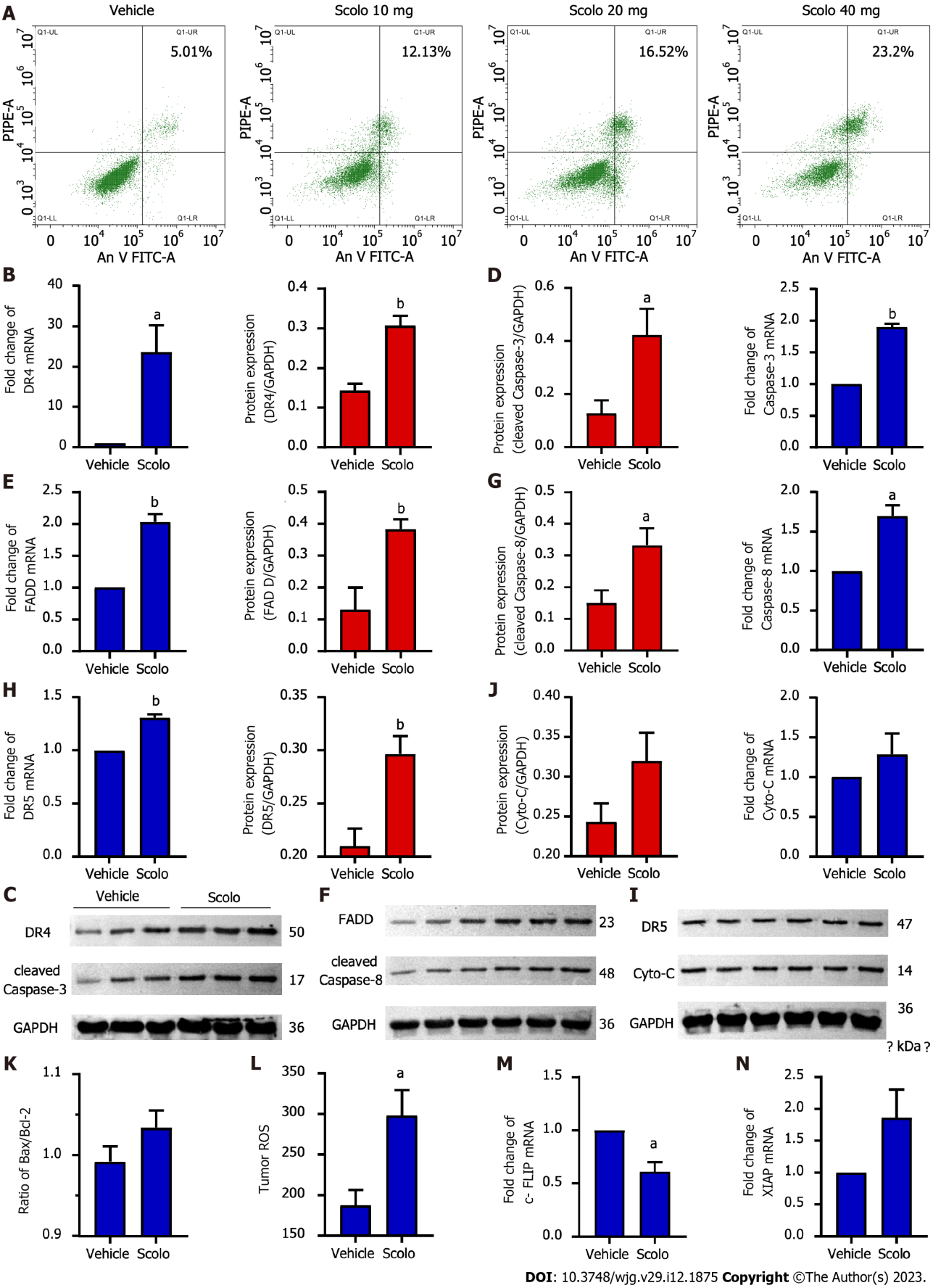


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Figure 5 Molecular docking of scolopentide and death receptor 4 and death receptor 5. A: Stereograms of the molecular docking of scolopentide and death receptor 4. Hydrogen bonds formed with amino acid residues of the receptor include LYS145, ALA201, PRO219, and CYS190; B: Stereograms of molecular docking of scolopentide and death receptor 5. Hydrogen bonds formed with amino acid residues of the receptor are TRP74, SER73, ER149, and TYR148.

We found that scolopentide may activate DR4 and DR5 and induce apoptosis. Flow cytometry suggested that apoptosis occurred in HepG2 cells after treatment with extracted scolopentide *in vitro* (Figure 6A). Hoechst staining showed the occurrence of apoptosis in HepG2 cells after treatment with synthetic scolopentide (Figure 3D). The CCK8 assay showed that scolopentide was significantly more cytotoxic in HepG2 cells than in L02 cells (Figure 4), which was similar to the effect of TRAIL, which selectively induced tumor cell apoptosis. Molecular docking also indicated that scolopentide binds well to TRAIL receptors (DR4 and DR5) (Figure 5). In our previous study, crude centipede peptides were also found to inhibit STAT3 protein phosphorylation[19], while some studies have suggested that dovitinib and sorafenib could overcome TRAIL resistance in HCC by inhibiting STAT3[50,51]. Consequently, animal experiments were used to further verify our hypothesis that scolopentide can stimulate the TRAIL pathway to induce apoptosis. In animal experiments, after treatment with synthetic scolopentide, the expression of DR4 and DR5, which are upstream of the TRAIL pathway, was clearly upregulated, especially the expression of DR4. The expression of FADD, caspase-8, and caspase-3, which are downstream of the TRAIL pathway, was also upregulated (Figure 6B-I). The animal experiments further confirmed the viewpoint that scolopentide induces apoptosis by activating DR4 and DR5.

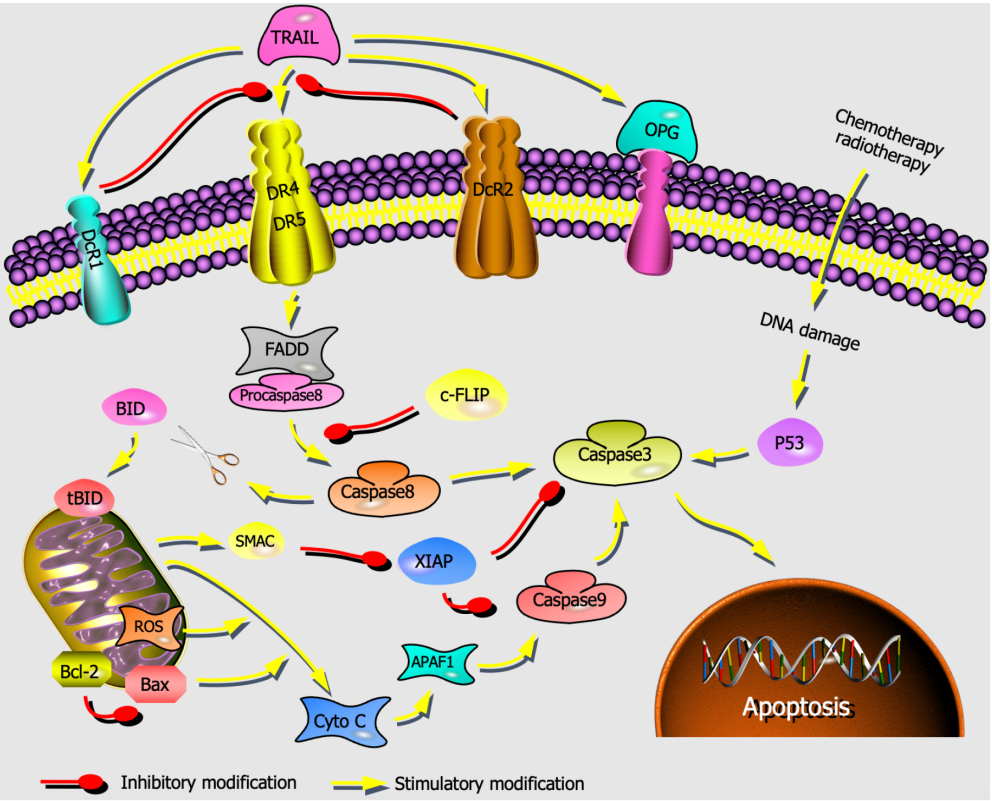
This process may be more related to the mitochondria-independent pathway, as Cyto-C and Bax/Bcl-2 showed only slight upregulation and insignificant differences (Figure 6I-K), which are key indicators in the mitochondria-dependent pathway[52]. ROS were significantly upregulated but could not cause Cyto-C release (Figure 6I, J and L). Thus, we hypothesized that ROS mainly acted as signal transduction factors of the TRAIL pathway rather than the mitochondrial-dependent pathway[49]. Additionally, c-FLIP was inhibited, but XIAP was slightly upregulated (Figure 6M and N). A negative feedback



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Figure 6 Antihepatoma mechanism of scolopentide. A: Flow cytometry suggested that apoptosis occurred in HepG2 cells after treatment with extracted scolopentide *in vitro*. B-I: Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting showed that the expression of DR4 (B and C), DR5 (H and I), FADD (E and F), caspase-8 (F and G), and caspase-3 (C and D) was significantly upregulated in the scolopentide group. DR4 was the most considerably

upregulated; I-K: qRT-PCR and western blotting showed that the expression of Cyto-C (I and J) and Bax/Bcl-2 (K) was not upregulated, which are key indicators of mitochondria dependence; L: Tumor ROS levels in the scolopentide group were higher than those in the vehicle group; M: c-FLIP, an inhibitory protein of caspase-8, was significantly downregulated in qRT-PCR; N: XIAP, an inhibitory protein of caspase-3, was insignificantly upregulated in qRT-PCR. DR4: Death receptor 4; DR5: Death receptor 5; FADD: Fas-associated death domain protein. n = 4 per group in qRT-PCR, n = 3 per group in Western blotting, n = 4 per group in ROS; [a]$P < 0.05$, [b]$P < 0.01$.



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Figure 7 Activation of the mitochondria-independent and mitochondria-dependent apoptosis pathways by tumor necrosis factor-related apoptosis-inducing ligand. The mitochondria-independent pathway (engaged through death receptors, activated caspase family directly) and mitochondria-dependent pathway (triggered through the Bcl-2 gene superfamily) are represented. TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; DR4: Death receptor 4; DR5: Death receptor 5; FADD: Fas-associated death domain protein; Cyto-C: Cytochrome c; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; ROS: Reactive oxygen species; c-FLIP: Cellular Fas associated death domain-like interleukin-1β converting enzyme inhibitory protein; XIAP: X-chromosome linked inhibitor-of-apoptosis protein.

mechanism of apoptosis may contribute to preventing excessive apoptosis in the body. Overall, our findings suggested that scolopentide may induce apoptosis in tumor cells, especially gastrointestinal tumors, by activating DR4 and DR5 and leading to the caspase cascade.

CONCLUSION

In summary, we obtained a small molecule polypeptide from *Scolopendra subspinipes mutilans L. Koch* with the strongest antitumor activity, especially for liver tumors. Mechanistically, scolopentide may induce tumor cell apoptosis by activating DR4 and DR5. Scolopentide is considered to be a promising drug candidate for cancer treatment, especially treatment of gastrointestinal tumors. Our studies are also expected to provide a reference for the extraction, purification and characterization of effective components in animal-derived medicines. Both extracted and synthesized scolopentide had anti-hepatoma activity. When HepG2 cells were cultured with two scolopentides for 48 h, a CCK8 assay showed that the IC_{50} values were 22.06 μg/mL (extracted scolopentide) and 237.726 μg/mL (synthesized scolopentide), which indicates that the antihepatoma activity of synthesized scolopentide was weaker than that of the extracted scolopentide. This may be due to the lack of dimensional folding configurations in the synthesized peptide during synthesis. Additionally, the TRAIL pathway was activated but decreased incrementally. Key areas of future research include investigating methods of modifying the spatial architecture of synthetic scolopentide, fully activating the TRAIL pathway and improving its

antihepatoma activity.

ARTICLE HIGHLIGHTS

Research background

Centipedes have been used to treat tumors for hundreds of years in China, while current studies rarely focus on hepatoma. The molecular identities of antihepatoma bioactive components in centipedes have not yet been extensively investigated. It is a challenge to isolate and characterize the effective components of centipedes due to limited peptide purification technologies for animal-derived medicines.

Research motivation

The antihepatoma components in centipedes remain unclear. We investigated the centipede components with the strongest antihepatoma activity to develop candidates for antihepatoma drugs.

Research objectives

To purify, characterize, and synthesize the bioactive components with the strongest antihepatoma activity from centipedes and determine the antihepatoma mechanism. To provide a reference for the extraction, purification and characterization of effective components for animal-derived medicines.

Research methods

An antihepatoma peptide (scolopentide) was isolated and identified from the centipede *scolopendra subspinipes mutilans* using a combination of enzymatic hydrolysis, a Sephadex G-25 column, and two steps of high-performance liquid chromatography. Additionally, the CCK8 assay was used to select the extracted fraction with the strongest antihepatoma activity. The molecular weight of the extracted scolopentide was characterized by quadrupole time-of-flight mass spectrometry, and the sequence was matched by using the Mascot search engine. Scolopentide was then synthesized using solid-phase peptide synthesis methods. The antihepatoma effects of extracted and synthetic scolopentide were confirmed *in vitro* and *in vivo*. Mechanistically, flow cytometry and Hoechst staining were conducted to confirm the occurrence of apoptosis. Molecular docking and CCK8 assays were performed to determine the relationship between scolopentide and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway. Reactive oxygen species assessment, quantitative real-time polymerase chain reaction and Western blot were used to further verify the hypothesis that scolopentide can stimulate the TRAIL pathway to induce apoptosis.

Research results

A small molecule polypeptide with the strongest antihepatoma activity was derived from *Scolopendra subspinipes mutilans L. Koch*. The molecular weight was 1018.997 Da, and the peptide sequence was RAQNHYCK. Both extracted and synthesized scolopentide had antihepatoma activity in a concentration-dependent manner. Mechanistically, scolopentide activated death receptor 4 (DR4) and DR5 and induced apoptosis in liver cancer cells by promoting the expression of Fas-associated death domain protein (FADD), caspase-8 and caspase-3 through a mitochondria-independent pathway.

Research conclusions

Scolopentide, an antihepatoma peptide, was isolated and identified from centipedes, which activated DR4 and DR5 and induced apoptosis through a mitochondria-independent pathway.

Research perspectives

Scolopentide is considered to be a promising drug candidate for cancer treatment, especially treatment of liver cancer. Ways in which to modify the spatial architecture of synthetic scolopentide, fully activate the TRAIL pathway and improve its antihepatoma activity will be key areas for future research.

ACKNOWLEDGEMENTS

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FOOTNOTES

Author contributions: Tian XF and Tian S contributed equally to this work, and they are co-corresponding authors; Tian XF, Tian S, Pei G, and Hu YX designed the study; Hu YX, Liu Z, Deng Z, Mei S, Yi C, Huang Z, and Feng T performed experiments; Pei G helped perform the high performance liquid chromatography and mass spectrum; Zhang Z performed data analyses; Zhou QH processed figures and tables; Hu YX wrote the manuscript; Tian XF, Zhou Q, and Zeng PH reviewed and edited the manuscript; all authors reviewed and approved the final version of the article.

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Institutional animal care and use committee statement: All animal experiments were conducted according to a protocol approved by the Ethics Review Committee of Experimental Animal Welfare at the Hunan University of Chinese Medicine (Approval No. LL2021040705).

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: Dataset available from the corresponding author at 003640@hnucm.edu.cn.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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ORIGINAL ARTICLE

Clinical Trials Study

Linked color imaging *vs* Lugol chromoendoscopy for esophageal squamous cell cancer and precancerous lesion screening: A noninferiority study

Zi-Xin Wang, Long-Song Li, Song Su, Jin-Ping Li, Bo Zhang, Nan-Jun Wang, Sheng-Zhen Liu, Sha-Sha Wang, Shuai Zhang, Ya-Wei Bi, Fei Gao, Qun Shao, Ning Xu, Bo-Zong Shao, Yi Yao, Fang Liu, En-Qiang Linghu, Ning-Li Chai

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Abstract

BACKGROUND

Lugol chromoendoscopy (LCE) has served as a standard screening technique in high-risk patients with esophageal cancer. Nevertheless, LCE is not suitable for general population screening given its side effects. Linked color imaging (LCI) is a novel image-enhanced endoscopic technique that can distinguish subtle differences in mucosal color.

AIM

To compare the diagnostic performance of LCI with LCE in detecting esophageal squamous cell cancer and precancerous lesions and to evaluate whether LCE can be replaced by LCI in detecting esophageal neoplastic lesions.

METHODS

In this prospective study, we enrolled 543 patients who underwent white light imaging (WLI), LCI and LCE successively. We compared the sensitivity and specificity of LCI and LCE in the detection of esophageal neoplastic lesions. Clinicopathological features and color analysis of lesions were assessed.

RESULTS

In total, 43 patients (45 neoplastic lesions) were analyzed. Among them, 36 patients (38 neoplastic lesions) were diagnosed with LCI, and 39 patients (41 neoplastic lesions) were diagnosed with LCE. The sensitivity of LCI was similar to that of LCE (83.7% *vs* 90.7%, $P = 0.520$), whereas the specificity of LCI was greater

than that of LCE (92.4% *vs* 87.0%, P = 0.007). The LCI procedure time in the esophageal examination was significantly shorter than that of LCE [42 (34, 50) s *vs* 160 (130, 189) s, P < 0.001]. The color difference between the lesion and surrounding mucosa in LCI was significantly greater than that observed with WLI. However, the color difference in LCI was similar in different pathological types of esophageal squamous cell cancer.

CONCLUSION

LCI offers greater specificity than LCE in the detection of esophageal squamous cell cancer and precancerous lesions, and LCI represents a promising screening strategy for general populations.

Key Words: Linked color imaging; Lugol chromoendoscopy; Esophageal squamous cell cancer; Precancerous lesions; Color difference

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Core Tip: Lugol chromoendoscopy (LCE) has served as the standard screening technique in high-risk patients with esophageal cancer. Nevertheless, LCE can induce esophageal spasms and chest pain and is associated with the risk of aspiration and iodine allergy. Therefore, LCE is not suitable for general population screening. Linked color imaging (LCI) is a novel image-enhanced endoscopy that can distinguish subtle differences in mucosal color. In this study, we compared the use of LCI and LCE in the screening of esophageal neoplastic lesions. Based on our analysis, we found that LCI is more specific than LCE and represents a promising screening strategy.

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INTRODUCTION

Esophageal cancer is the seventh most common cancer and the sixth leading cause of cancer-related deaths worldwide[1]. Most patients with esophageal cancer have progressed to an advanced stage when diagnosed, with poor quality of life and an overall 5-year survival rate of less than 20%[2,3]. Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer worldwide, representing greater than 85% of all esophageal cancer cases[4,5]. Curative resection using endoscopic mucosal resection or endoscopic submucosal dissection is possible for lesions with high-grade intraepithelial neoplasia and most T1 tumors, offering less trauma, faster recovery and fewer complications[6]. Therefore, early diagnosis is critical for treatment success and prognostic improvement. Early identification and timely intervention of esophageal cancer or precancerous lesions are of great significance to delay the progression of the disease, improve the prognosis and improve the quality of life.

However, under white light imaging (WLI) endoscopy, early neoplastic lesions are easily missed due to the presence of small lesion areas and subtle differences in the surrounding mucosa color[7]. Historically, Lugol chromoendoscopy (LCE) has served as the standard diagnostic technique given its higher detection rate[8-12]. However, its specificity is low due to light staining under conditions of inflammation, which has been demonstrated in previous studies[13-15]. Furthermore, LCE occasionally induces esophageal spasms and chest pain and is associated with the risk of aspiration and iodine allergy[16,17].

With the advent of image-enhanced endoscopy, many studies have confirmed its efficacy in diagnosing upper gastrointestinal neoplasms[13,18]. The LASEREO system (Fujifilm Corporation, Tokyo, Japan), a new linked color imaging (LCI) technology, was recently developed. LCI images are illuminated with white light and short wavelength narrow-band light in an appropriate proportion simultaneously to realize the simultaneous expansion and contraction of colors. This procedure facilitates enhancement of the red and white colors and makes it easier for the operators to identify the subtle differences in mucosal color[18-21]. In our clinical work, it was found that LCI significantly improved the ability to identify lesions. In a large-scale randomized comparative study, Ono *et al*[20] reported the superiority of LCI over WLI in detecting neoplastic lesions in the upper gastrointestinal tract. Previous studies reported the usefulness of LCI for esophageal neoplasm detection and

assessments of invasive depth[20-24]. Although Yi *et al*[25] compared LCI and LCE in the diagnosis of multiple primary esophageal cancers, no prospective study has compared the diagnosability of LCI with LCE in screening esophageal neoplastic lesions to date. Therefore, we conducted a prospective noninferiority study to compare the efficacy of non-magnifying LCI and LCE in detecting and diagnosing ESCC and precancerous lesions. We evaluated whether LCE can be replaced by LCI in detecting esophageal neoplastic lesions for the general population.

MATERIALS AND METHODS

Study design

This was a single-center, prospective, registered clinical study (No. ChiCTR2100045636) conducted at the First Medical Center of Chinese PLA General Hospital that compared the detection of ESCC and precancerous lesions using LCI *vs* LCE after a conventional white light examination. Endoscopic biopsy is considered the gold standard for ESCC detection. The study was approved by the medical ethics committee of the Chinese PLA General Hospital (Beijing, China), and informed consent forms were obtained from all patients.

Patients

The regional screening studies of esophageal cancer in China revealed that endoscopic screening starting at 40 years of age was cost-effective[26,27]. We recommended 40 years as the beginning age for screening in the Chinese consensus[28]. The following inclusion criteria were employed: (1) Age > 40-year-old; and (2) Provision of written consent to participate. The exclusion criteria were as follows: (1) Contraindication for upper gastrointestinal endoscopy or general anesthesia; (2) Iodine allergy or hyperthyroidism; and (3) Previous surgical resection or chemotherapy, radiotherapy or chemoradiotherapy for ESCC (as these procedures may influence the mucosal surface, which is important for detecting these lesions).

Diagnostic strategies

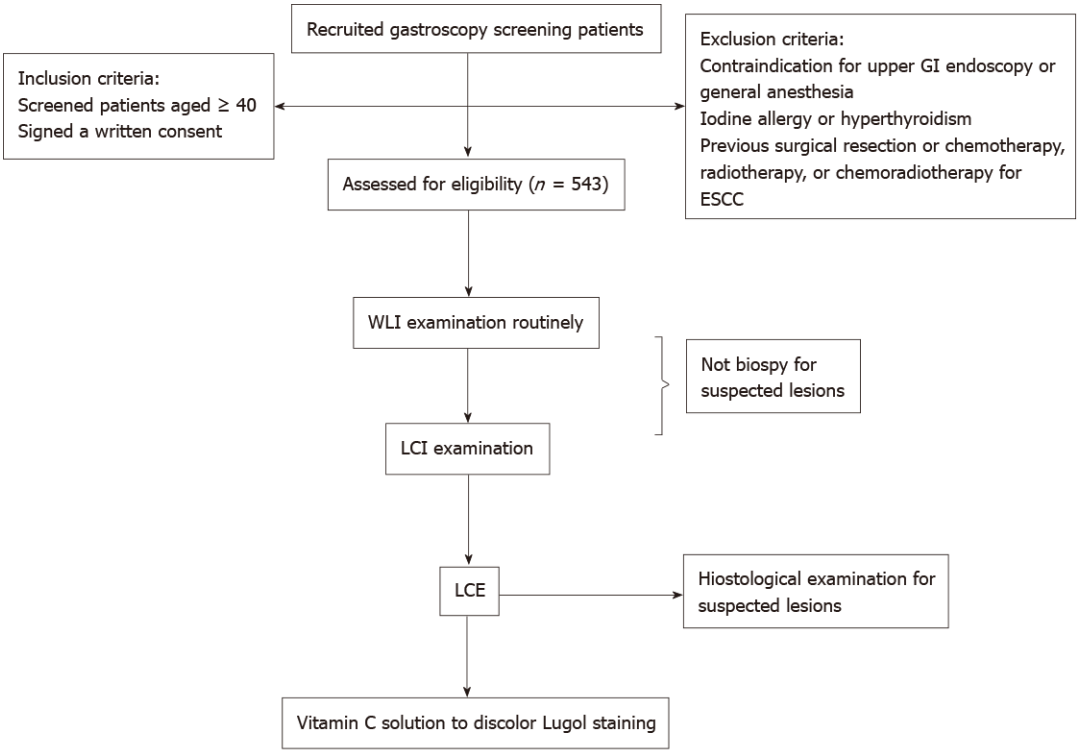
This study used an upper EG-590WR scope (Fujifilm) for gastroscopy. The specific examination procedure was performed as follows. First, WLI examination was performed, and all detected lesions were described. Second, LCI examination was performed, and all lesions detected were recorded in the report (another researcher) but not biopsied immediately to avoid bleeding that could influence the Lugol examination. Third, LCE was performed (2% Lugol dye was spread over the entire esophagus using a spray catheter except near the upper esophageal sphincter given the risk of aspiration), and we biopsied the suspected lesions detected by LCE. Finally, vitamin C solution was used to discolor the Lugol-stained mucosa[29], and lesions suspected only in LCI mode were biopsied based on the recorded location. All suspected lesions were biopsied, and the modality (only LCI, only LCE, or both LCI and LCE) that detected each lesion was indicated in the report. A flow chart of the study is described in Figure 1. All endoscopic operators were experienced and had completed at least 1000 endoscopies. The diagnosis modality (LCI or/and LCE), size, location and macroscopic type (Paris classification[30]) of each detected lesion were recorded and described after examination.

Outcome

The diagnostic criteria for intraepithelial neoplasia and ESCC proposed by the Vienna Classification are as follows: Low-grade intraepithelial neoplasia (LGIN); high-grade intraepithelial neoplasia (HGIN), ESCC and negative for neoplasia, including chronic esophagitis[31]. The primary outcome was the sensitivity and specificity of the diagnostic strategies to detect squamous cell neoplastic lesions (LGIN, HGIN and/or ESCC) according to histology. Endoscopically suspicious ESCC lesions and precancerous lesions were defined as follows: (1) The presence of a reddish color change with a rough mucosal surface in WLI (Figure 2A); (2) The presence of a well-demarcated red or red-orange color region in LCI (Figure 2B)[21,22]; and (3) The presence of a well-demarcated unstained area ≥ 5 mm in diameter or a pink color area after iodine staining given that a Lugol-voiding lesion is more likely to be neoplastic with increasing size (Figure 2C)[32,33].

All suspected lesions were pathologically evaluated by biopsy to determine their neoplastic nature. If the lesion was confirmed to be neoplastic, it was a true positive for the detection modality. Conversely, for non-neoplastic lesions, an unconfirmed suspected lesion was defined as a false positive for the detection modality.

Major outcomes included sensitivity and specificity. The secondary outcome was procedure time. It was possible to determine the length of the imaging operation using a clock linked to the endoscopic equipment. The LCI procedure time was defined as the total examination time from the epiglottis to the dentate line in the image files. The LCE procedure time was defined as the examination time from spraying Lugol dye to complete discoloration.



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Figure 1 Flow chart of study design. ESCC: Esophageal squamous cell carcinoma; GI: Gastrointestinal; LCE: Lugol chromoendoscopy; LCI: Linked color imaging; WLI: White light imaging.

Sample size

In this study, we calculated the required sample size for a noninferiority test using the PASS 15.0.5 sample size software (NCSS, LLC, Kaysville, UT, United States). We regarded LCE as the reference endoscopy technique, and sensitivity was assumed to be 90.0%[13,14]. A sensitivity of 80.0% would be a clinically adequate diagnostic value for screening endoscopy for ESCC. The sample size required was 535 patients using the Z test with pooled variance for a statistical power of 90% with statistical significance defined as $P < 0.05$ ($\alpha = 0.05$ and $\beta = 0.10$).

Color analysis

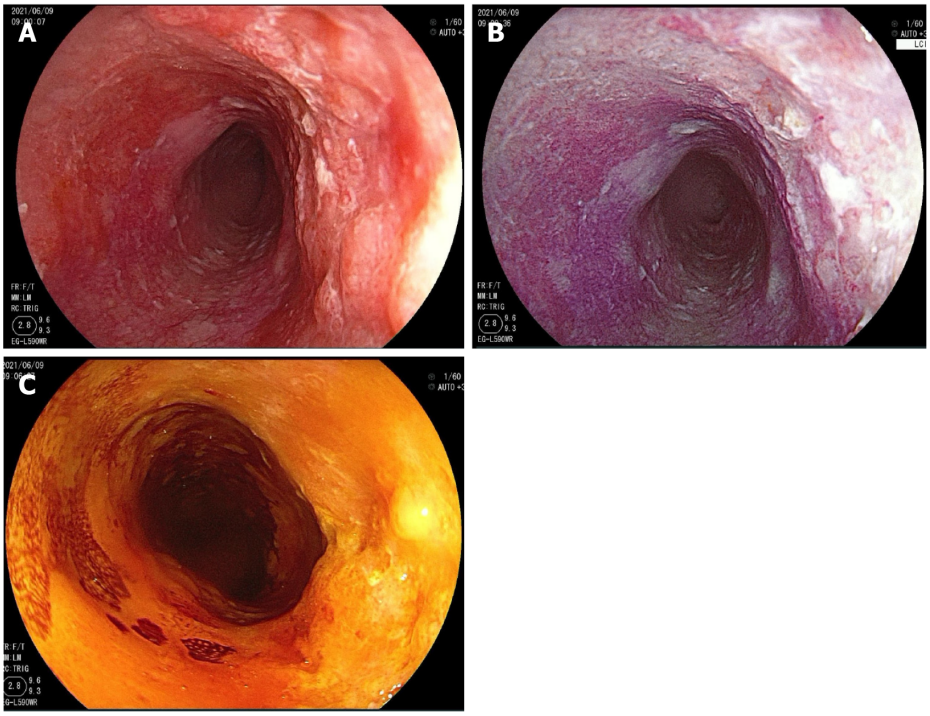
Color analysis was performed using Adobe Photoshop CC2017 (Adobe Systems Inc., San Jose, CA, United States). First, regions of interest of the lesion mucosa and surrounding mucosa were selected in WLI and LCI, separately (Figure 3). Second, the Commission International de l'Eclairage - L*a*b* color space was used to evaluate the mean color value in the regions of interest[34]. The three-dimensional color parameters L* (black to white; range: 0 to + 100), a* (green to red; range: 128 to + 127) and b* (blue to yellow; range: 128 to + 127) were used to define the color value. In Photoshop, L*, a* and b* represent color scores (Lab color unit). Third, the color difference between the lesion and the surrounding mucosa (ΔE) was represented by the distance connecting the two points:

$$\Delta E = \sqrt{\{(\Delta L*)2 + (\Delta a*)2 + (\Delta b*)2\}}$$

To reduce the influence of color difference depending on imaging conditions, we chose similar distance and angle images in each mode to calculate color values. We calculated the ΔE of each image in LCI and WLI. In addition, we calculated the ΔE of different neoplastic pathologies in LCI and compared them.

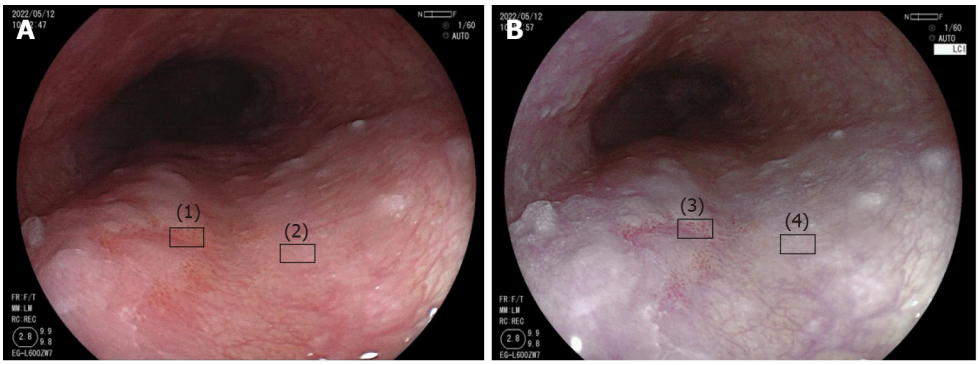
Statistical analysis

All analyses were performed using SPSS 26.0 statistical software (IBM Corp., Armonk, NY, United States). The characteristics and diagnostic yields of patients were presented as percentages (%) or medians (interquartile range), and the color difference variables were expressed as the mean ± SD. Continuous variables were compared using the student's t-test or the Mann-Whitney U test, whereas categorical variables were compared using Pearson's χ^2 test or Fisher's exact test. A two-tailed P value of < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by the staff of the Department of Epidemiology and Statistics from the First Medical Center of PLA General Hospital (Beijing, China).



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Figure 2 A single esophageal squamous cell carcinoma lesion visualized under the three detection methods. A: Under white light imaging; B: Under linked color imaging; C: Under Lugol chromoendoscopy.



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Figure 3 Method to measure the color difference between neoplastic and non-neoplastic lesions detected by white light imaging and linked color imaging. A: Neoplastic lesion (1) and non-neoplastic lesion (2) detected by white light imaging; B: Neoplastic lesion (3) and non-neoplastic lesion (4) detected by linked color imaging.

RESULTS

Participants

From March 2021 to May 2022, a total of 543 patients were enrolled in our study (Figure 1). Among them, a total of 141 suspected lesions were identified in 115 patients. Of these lesions, 45 neoplastic lesions (17 ESCC, 3 HGIN and 25 LGIN) were histologically confirmed in 43 (7.8%) patients (Table 1). Among the 45 lesions, 38 lesions (17 ESCC, 3 HGIN and 18 LGIN) were endoscopically suspicious during LCI examination; further, 41 lesions (17 ESCC, 3 HGIN and 21 LGIN) were endoscopically suspicious during LCE examination. Regarding the diagnostic modality of the 45 lesions, 34 (75.6%) lesions were identified by LCI and LCE compared to 4 (8.9%) lesions that were detected only by LCI and 7 (15.6%) lesions that were detected only by LCE.

Diagnostic performance of LCI and LCE for neoplastic lesions

In LCI mode, 74 suspected lesions underwent histological examination, and 38 lesions were confirmed histologically in 36 patients. In LCE mode, 104 suspected lesions were detected, and 41 lesions were

Table 1 Clinicopathological features of the patients

Item	*n*	% (*n*/*n* total)
Patients with suspected lesions[1]	115	21.2 (115/543)
Inflammation	72	13.2
LGIN	25	4.6
HGIN	3	0.5
ESCC	15	2.7
Suspected lesions, including non-neoplastic	141	-
Neoplastic lesions[2]	45	31.9 (45/141)
LGIN	25	17.7 (25/141)
HGIN	3	2.1 (3/141)
ESCC	17	12.1 (17/141)
Neoplastic lesions detected with LCI		
LGIN	18	72.0 (18/25)
HGIN	3	100 (3/3)
ESCC	17	100 (17/17)
Neoplastic lesions detected with LCE		
LGIN	21	84.0 (21/25)
HGIN	3	100 (3/3)
ESCC	17	100 (17/17)
Neoplastic lesions detected by different diagnosis modality		
Only LCI	4	8.9 (4/45)
Only LCE	7	15.6 (7/45)
Both LCI and LCE	34	75.6 (34/45)
Neoplastic lesions per patient, mean (SD)	0.08 (0.29)	

[1]Suspected lesions included neoplastic lesions and false positives (lesions detected but not confirmed to be neoplastic after histological examination).
[2]Neoplastic lesions confirmed by histological examination.
ESCC: Esophageal squamous cell carcinoma; HGIN: High-grade intraepithelial neoplasia; LCE: Lugol chromoendoscopy; LCI: Linked color imaging; LGIN; Low-grade intraepithelial neoplasia; SD: Standard deviation.

histologically confirmed in 39 patients. In Table 2, the sensitivity, specificity, positive predictive value and negative predictive value for LCI for the diagnosis of ESCC/HGIN/LGIN were 83.7%, 92.4%, 48.6% and 98.5%, respectively. The sensitivity, specificity, positive predictive value and negative predictive value for LCE were 90.7%, 87.0%, 37.5% and 99.1%, respectively. No significant differences in sensitivity, positive predictive value and negative predictive value were noted between LCI and LCE. However, the specificity of LCI was significantly higher than that of LCE (92.4% *vs* 87.0%, $P = 0.0023$).

Characteristics of the neoplastic lesions detected by LCI and LCE

The characteristics of the neoplastic lesions detected by LCI and LCE of the respective comparison groups are shown in Table 3. No significant differences in lesion location, size, morphologic type or pathology were noted between the two modality groups. However, the procedure time was significantly shorter for the LCI group compared with the LCE group [42 (34, 50) s *vs* 160 (130, 189) s, $P < 0.001$].

All detected neoplastic lesions were further divided into three subgroups according to the different diagnosis modalities: LCI only group (detected by LCI only, $n = 4$ lesions), LCE only group (detected by LCE only, $n = 7$ lesions), and LCI + LCE group (detected by both LCI and LCE, $n = 34$ lesions). We compared the clinicopathological features of the three groups in Supplementary Table 1. The lesion location, morphologic type and pathology were comparable among the three groups. However, the lesion sizes of the three groups were significantly different: 20 (55.8%) lesions in the LCI + LCE group were > 20 mm; 3 (75%) lesions in the LCI only group were ≤ 10 mm; and 7 (100%) lesions in the LCE only group were ≤ 10 mm ($P = 0.016$). We further compared the lesion size in the LCI only group and

Table 2 Esophageal neoplastic lesions (non-inflammation) detected using linked color imaging and Lugol chromoendoscopy

Parameter	LCI	LCE	*P* value
Sensitivity	83.7 (36/43)	90.7 (39/43)	0.520
Specificity	92.4 (462/500)	87.0 (435/500)	0.007[1]
PPV	48.6 (36/74)	37.5 (39/104)	0.166
NPV	98.5 (462/469)	99.1 (435/439)	0.549

[1]$P < 0.01$.

Data are presented as % (n/n total). LCE: Lugol chromoendoscopy; LCI: Linked color imaging; NPV: Negative predictive value; PPV: Positive predictive value.

Table 3 Clinicopathological features of neoplastic lesions (non-inflammation) detected by linked color imaging and Lugol chromoendoscopy, *n* (%)

Item		Detected by LCI, *n* = 36 patients/38 lesions	Detected by LCE, *n* = 39 patients/41 lesions	*P* value
Lesion location				0.969
Upper		3 (7.9)	3 (7.3)	
Middle		11 (28.9)	11 (26.8)	
Lower		24 (63.2)	27 (65.9)	
Morphologic type				0.902
I		1 (2.6)	1 (2.4)	
IIa		9 (23.7)	8 (19.5)	
IIb		18 (47.4)	24 (58.5)	
IIc		9 (23.7)	7 (17.1)	
III		1 (2.6)	1 (2.4)	
Size in mm				0.814
≤ 10		14 (36.8)	18 (43.9)	
10-20		3 (7.9)	3 (7.3)	
> 20		21 (55.3)	20 (48.8)	
Pathology				0.998
LGIN		18 (47.4)	21 (51.2)	
HGIN		3 (7.9)	3 (7.3)	
ESCC	EP/LPM	3 (7.9)	3 (7.3)	
	MM/SM1	1 (2.6)	1 (2.4)	
	≥ SM2	13 (34.2)	13 (31.7)	
Procedure time (s), median (IQR)		42 (34, 50)	160 (130, 189)	< 0.001[1]

[1]P value < 0.01.

EP: Epithelial; ESCC: Esophageal squamous cell carcinoma; HGIN: High-grade intraepithelial neoplasia; IQR: Interquartile range; LCE: Lugol chromoendoscopy; LCI: Linked color imaging; LGIN: Low-grade intraepithelial neoplasia; LPM: Lamina propria mucosa; MM: Muscularis mucosa; SM1: Submucosa invading ≤ 200 μm below the inferior margin of the muscularis mucosa; SM2: Submucosa invading > 200 μm below the inferior margin of the muscularis mucosa.

LCE only group, and the difference was not statistically significant ($P = 0.364$).

Color analysis

Of the 45 neoplastic lesions, 30 lesions had endoscopic images with clear visibility. Color analysis was conducted in the 30 lesions. Supplementary Table 2 compares the color difference between the lesion

and surrounding mucosa in WLI and LCI. We found that the ΔE between the lesions and the surrounding mucosa was significantly higher in LCI compared with WLI (21.20 ± 9.79 *vs* 15.92 ± 7.50, respectively, $P = 0.023$). For the three-dimensional color parameters of L*a*b*, Δa^* was higher in LCI than in WLI (12.43 ± 10.00 *vs* 4.97 ± 6.96, respectively, $P = 0.001$), and ΔL^* and Δb^* were similar in LCI and WLI. We further analyzed the color difference between the lesion and surrounding mucosa in different neoplastic pathologies (LGIN, HGIN and ESCC) in LCI mode, as shown in Supplementary Table 3. However, no significant differences in ΔE, ΔL^*, Δa^* and Δb^* were noted among the three pathological types.

DISCUSSION

In this prospective study, we demonstrated that the sensitivity and specificity of LCI to detect ESCC and/or precancerous lesions (LGIN or/and HGIN) are acceptable. The specificity of LCI is higher than that of LCE, and the differences in sensitivity between the two modes were not obvious.

Previous studies have reported the utility of LCE in the detection of esophageal cancer[7,10-12]. LCE has served as the reference technique in patients at high risk for esophageal cancer[8,9]. Nevertheless, LCE is not suitable for general population screening given its side effects[16,17]. As a type of enhanced endoscopy, some studies have shown that narrow-band imaging is comparable to LCE in detecting esophageal cancer due to its excellent sensitivity and specificity[13-15]. Narrow-band imaging is typically combined with magnifying endoscopy, and the requirements for hospitals and operators are relatively high. LCI is a novel enhanced endoscopic technique, and Ono *et al*[20] reported that the detection rate of LCI in the diagnosis of neoplastic lesions in the upper gastrointestinal tract is 1.67 times higher than that of WLI. Yi *et al*[25] compared LCI and LCE in the detection of multiple primary esophageal cancers in primary ESCC patients and found that both modalities exhibited great value for multiple primary esophageal cancers. However, no study has compared their screening ability. To the best of our knowledge, this was the first prospective study to compare the effectiveness of LCI and LCE in screening esophageal cancer and precancerous lesions.

The present results showed that LCI is significantly more specific than LCE (92.4% *vs* 87.0%, $P = 0.0023$), and both modes exhibited high sensitivity for detecting neoplastic lesions (LGIN/HGIN/ESCC) (83.7% *vs* 90.7%, $P = 0.520$). The lesions were all pathologically diagnosed as LGIN (7 lesions in LCI and 4 in LCE). Therefore, our study results were consistent with previously published data reporting that the sensitivity of narrow-band imaging and Lugol for the diagnosis of ESCC and/or HGIN was approximately 100%[13,14]. The LCI only group included a lesion approximately 4 cm in length, and no unstained areas were observed. Based on the endoscopic findings, we thought that it was an erosive mucosal lesion caused by gastric acid reflux. Our results indicate that the capability of LCI to detect neoplastic lesions is acceptable.

The LCI technique is convenient for clinical endoscopists to use with the Fujifilm system because the imaging modes can easily be switched during the examination. Thus, the process is not as time-consuming as iodine staining. In our research, the median LCI procedure time was significantly shorter than for LCE (42 s *vs* 160 s, $P < 0.001$). In addition, Lugol solution can irritate the mucosa and may cause many side effects (chemical esophagitis, laryngitis, bronchopneumonia, chest pain, esophagospasm, gastritis and hypersensitivity)[16,17]. In clinical practice, the upper end of the Lugol staining site is generally less than 20 cm from the incisor given the high risk of solution aspiration. Therefore, it is difficult to detect cervical esophageal lesions, which reduces the detection of synchronous or metachronous neoplastic lesions. Therefore, the LCI technique is more useful for the diagnosis of ESCC or precancerous lesions given the mild mucosal irritation.

After color analysis, we found that the color difference between the lesion and the surrounding mucosa was greater for LCI compared with WLI. The results demonstrated that the lesion was more visible in LCI mode, which is consistent with previous studies[18,19,21,22]. Kobayashi *et al*[21] investigated the relationship between color information and the invasion depth of ESCC in LCI mode. They found that the color difference was greater in muscularis mucosa/submucosa invading ≤ 200 μm below the inferior margin of the muscularis mucosa or deeper lesions compared with epithelium and lamina propria mucosa lesions using LCI. However, they did not compare the color differences between ESCC and precancerous lesions. In our study, we further compared the color difference of different neoplastic pathologies in LCI mode, and the differences among LGIN, HGIN and ESCC were not significant. Notably, Tsunoda *et al*[35] reported a case using LCI and blue laser imaging with Lugol staining to provide an accurate diagnosis of ESCC and squamous intraepithelial neoplasia. Therefore, whether LCI and blue laser imaging combined with Lugol staining can be used to evaluate esophageal neoplastic lesions before endoscopic treatment deserves further study.

There are some limitations in our study. First, the number of lesions, especially neoplastic lesions, was low. Second, LCI and LCE can be performed sequentially during the same endoscopy procedure, but it is impossible to perform them in the reverse order. Thus, we were unable to perform a random crossover trial. Third, the images obtained in this study included two arbitrary regions of interest in the lesion mucosa and surrounding mucosa, which may have sampling errors and affected the

measurement results. However, for the WLI and LCI modes, we choose similar distance and angle images to calculate color values to reduce the influence of color differences based on various conditions. In the future, we need to conduct a multicenter study and collect more neoplastic lesions to further evaluate the usefulness of LCI. Further evaluation of the validity of LCI in diagnosing the depth of invasion of ESCC is also warranted.

CONCLUSION

Our study confirmed that LCI is efficient and specific for the surveillance of ESCC without causing discomfort. In the future, LCI, as a promising screening strategy, could replace LCE in the screening of esophageal neoplastic lesions in the general population.

ARTICLE HIGHLIGHTS

Research background

Lugol chromoendoscopy (LCE) has served as a standard screening technique in high-risk patients with esophageal cancer. Nevertheless, LCE is not suitable for the general population screening given its side effects. Linked color imaging (LCI) is a novel image-enhanced endoscopic technique that can distinguish subtle differences in mucosal color. It would be beneficial for the general population if LCE can provide similar diagnostic performance to LCI.

Research motivation

We compared the diagnostic performance of LCI with LCE in detecting esophageal squamous cell carcinoma (ESCC) and precancerous lesions. If LCI can replace LCE in detecting esophageal neoplastic lesions, it would be useful for esophageal screening in the general population.

Research objectives

As a novel image-enhanced endoscopic technique, LCI has been confirmed to be superior to white light imaging (WLI) in detecting neoplastic lesions in the upper gastrointestinal tract. We aimed to confirm that the diagnostic performance of LCI is comparable to LCE for the surveillance of ESCC.

Research methods

This was a single-center, prospective, registered clinical study. In this noninferiority study, we prospectively enrolled 543 patients who underwent WLI, LCI and LCE successively. We compared the sensitivity and specificity of LCI and LCE in the detection of esophageal neoplastic lesions. We further used L*a*b* color space to evaluate the color differences of LCI.

Research results

In total, 43 patients were analyzed. The sensitivity of LCI was similar to that of LCE, whereas the specificity of LCI was greater than that of LCE. The LCI procedure time in the esophageal examination was significantly shorter than that of LCE. However, the color difference in LCI was similar in different pathological types.

Research conclusions

Our study showed that LCI is efficient and specific for the surveillance of ESCC without causing discomfort. In the future, LCI, as a promising screening strategy, could replace LCE in the screening of esophageal neoplastic lesions in the general population.

Research perspectives

Because of the low detection rate of esophageal cancer, we were only able to enroll a limited number of neoplastic lesions. In the future, we need to conduct a multicenter study and collect more neoplastic lesions to further evaluate the usefulness of LCI. Further evaluation of the validity of LCI in diagnosing the depth of invasion of ESCC is also warranted.

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FOOTNOTES

Author contributions: Wang ZX and Li LS contributed equally to this work; Chai NL and Linghu EQ contributed to study conception and design; Wang ZX and Li LS contributed to manuscript drafting; Li LS, Su S, Li JP, Wang NJ, Zhang B, Liu SZ, Zhang S, Wang SS, Bi YW, Gao F, Shao Q, Xu N, Shao BZ, Yao Y, and Liu F contributed to data acquisition; Chai NL, Linghu EQ, Li LS and Su S contributed to critical revision of the article for important intellectual content; and all authors issued final approval for the version to be submitted.

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Institutional review board statement: The study was reviewed and approved by the ethics committee of the Chinese PLA general hospital (Approval No. of Ethics Committee: S2021-145-01).

Clinical trial registration statement: This study was registered in the Chinese clinical trial database (chictr.org) with the number ChiCTR2100045636.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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REVIEW

Application of nanotechnology in reversing therapeutic resistance and controlling metastasis of colorectal cancer

Sheng-Nan Ren, Zhan-Yi Zhang, Rui-Jie Guo, Da-Ren Wang, Fang-Fang Chen, Xue-Bo Chen, Xue-Dong Fang

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Abstract

Colorectal cancer (CRC) is the most common digestive malignancy across the world. Its first-line treatments applied in the routine clinical setting include surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. However, resistance to therapy has been identified as the major clinical challenge that fails the treatment method, leading to recurrence and distant metastasis. An increasing number of studies have been attempting to explore the underlying mechanisms of the resistance of CRC cells to different therapies, which can be summarized into two aspects: (1) The intrinsic characters and adapted alterations of CRC cells before and during treatment that regulate the drug metabolism, drug transport, drug target, and the activation of signaling pathways; and (2) the suppressive features of the tumor microenvironment (TME). To combat the issue of therapeutic resistance, effective strategies are warranted with a focus on the restoration of CRC cells' sensitivity to specific treatments as well as reprogramming impressive TME into stimulatory conditions. To date, nanotechnology seems promising with scope for improvement of drug mobility, treatment efficacy, and reduction of systemic toxicity. The instinctive advantages offered by nanomaterials enable the diversity of loading cargoes to increase drug concentration and targeting specificity, as well as offer a platform for trying the combination of different treatments to eventually prevent tumor recurrence, metastasis, and reversion of therapy resistance. The present review intends to summarize the known mechanisms of CRC resistance to chemotherapy, radiotherapy, immunotherapy, and targeted therapy, as well as the process of metastasis. We have also emphasized the recent application of nanomaterials in combating therapeutic resistance and preventing metastasis either by combining

with other treatment approaches or alone. In summary, nanomedicine is an emerging technology with potential for CRC treatment; hence, efforts should be devoted to targeting cancer cells for the restoration of therapeutic sensitivity as well as reprogramming the TME. It is believed that the combined strategy will be beneficial to achieve synergistic outcomes contributing to control and management of CRC in the future.

Key Words: Colorectal cancer; Therapeutic resistance; Nanotechnology; Drug delivery system; Combined treatment

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Core Tip: Mechanisms of Colorectal cancer (CRC) cell resistance can be attributed to tumoral and environmental factors, the former of which includes gene expression alteration, signaling pathway activation, metabolic rewiring, and the later refers to complicated adaptions regarding cancer associated fibroblast, immune cells, hypoxic conditions. Taking efforts to conquer therapeutic resistance is imperative to improve CRC patients` survival. Nanotechnology possesses distinct advantages to increase specificity of treatment and realize codelivery of multiple drugs, which facilitates to restore sensitivity to antitumor therapy, and modulate suppressive tumor microenvironment to stimulatory environment.

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INTRODUCTION

Colorectal cancer (CRC) is the most common malignancy of the digestive tract, accounting for the death of 0.9 million people across the world in 2020 alone[1]. Surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are among the present first-line strategies applied to treat CRC patients and control their recurrence. Surgery is accepted as the primary treatment for CRC, especially for early stage CRC, as it eradiates the local lesions thoroughly in a timely manner[2]. Five-Flurouracil (5-FU)- and oxaliplatin-based chemotherapy contribute to the control of local recurrence and metastasis of CRC after surgery. As for advanced CRC, radiotherapy and neoadjuvant chemotherapy are important modalities that have been reported to contribute to prolonging the patients' survival[3,4]. In addition, cetuximab and immunocheckpoint inhibitor (ICI)-based therapy can be administrated to EGFR-overexpressed CRC or microsatellite instability (MSI)-subtype CRC so as to achieve greater survival benefits[5, 6]. However, despite the availability of comprehensive treatment against CRC, resistance to therapy and metastasis continue to be reported during or after the treatment, often resulting in relapse or treatment failure.

Chemotherapy resistance is a common cause of CRC treatment failure, and the intrinsic and acquired resistance of tumor cells have been implicated as the major causes[7]. Aberrant metabolism and change in the drug targets have generally been reported to be responsible for the 5-FU resistance of CRC cells developed during the treatment duration[8,9]. The development of resistance to radiotherapy is usually related to hypoxia and the adaption of cancer stem cells (CSC) that play a pivotal role in the regulation of survival and proliferation of CRC[10]. In addition, the tumor microenvironment (TME) is involved in the revolution and development of tumor cells to resist attacks from drugs and immune cells[11]. Many efforts have been made toward enhancing the treatment efficacy after resistance is developed, such as by increasing the drug dosage or combining multiple treatments. However, the improvements in response and the overall survival rate remain unsatisfactory due to the issues of increased toxicity and poor tolerance. Consequently, more effective drug-delivery methods and therapeutic strategies are urgently warranted to overcome the barrier of the suppressive microenvironment as well as to restore the sensitivity of CRC cells to the prescribed treatment.

Thanks to the unique advantages offered by nanotechnology, it has drawn the great attention of researchers and clinicians in their quest to conquer the issue of treatment resistance of CRC with its valuable potential. Drug-delivery systems based on nanomaterials can simultaneously carry a variety of components and increase the selectivity of the systemic treatment and the local drug concentration through active and passive targeting effects[12-14]. This approach not only delivers cytotoxic drugs but also delivers nucleic acid molecules, thereby realizing the combined application of chemotherapy,

immunotherapy, gene therapy, and other treatments[15-17]. In addition, several nanomaterials possess anticancer potential or can hence be utilized as radiotherapy sensitizers and they generate a photothermal or photodynamic effect that can facilitate tumor control[18,19]. When used either intraoperatively or postoperatively, nanomaterial-based treatment can be applied to remodel the local microenvironment and activate the innate and adaptive immune responses toward controlling tumor growth and metastasis, which, in turn, generate immune-memory effects called immunomodulation[20-22]. Collectively, nanotechnology is expected to solve the treatment resistance of CRC as a valuable alteration to realize the effective control of tumor recurrence and metastasis. In this review, we have illustrated the mechanisms of therapeutic resistance of CRC cells to various treatments, including both tumoral and environmental factors (Figure 1). Meanwhile, recent advancements in nanotechnology utilized in CRC treatment aimed at restoring tumor sensitivity and reversing resistance will be further introduced, along with the challenges and perspectives for nanotechnology-based CRC treatment.

MECHANISMS OF THERAPEUTIC RESISTANCE AND METASTASIS

Chemotherapy resistance

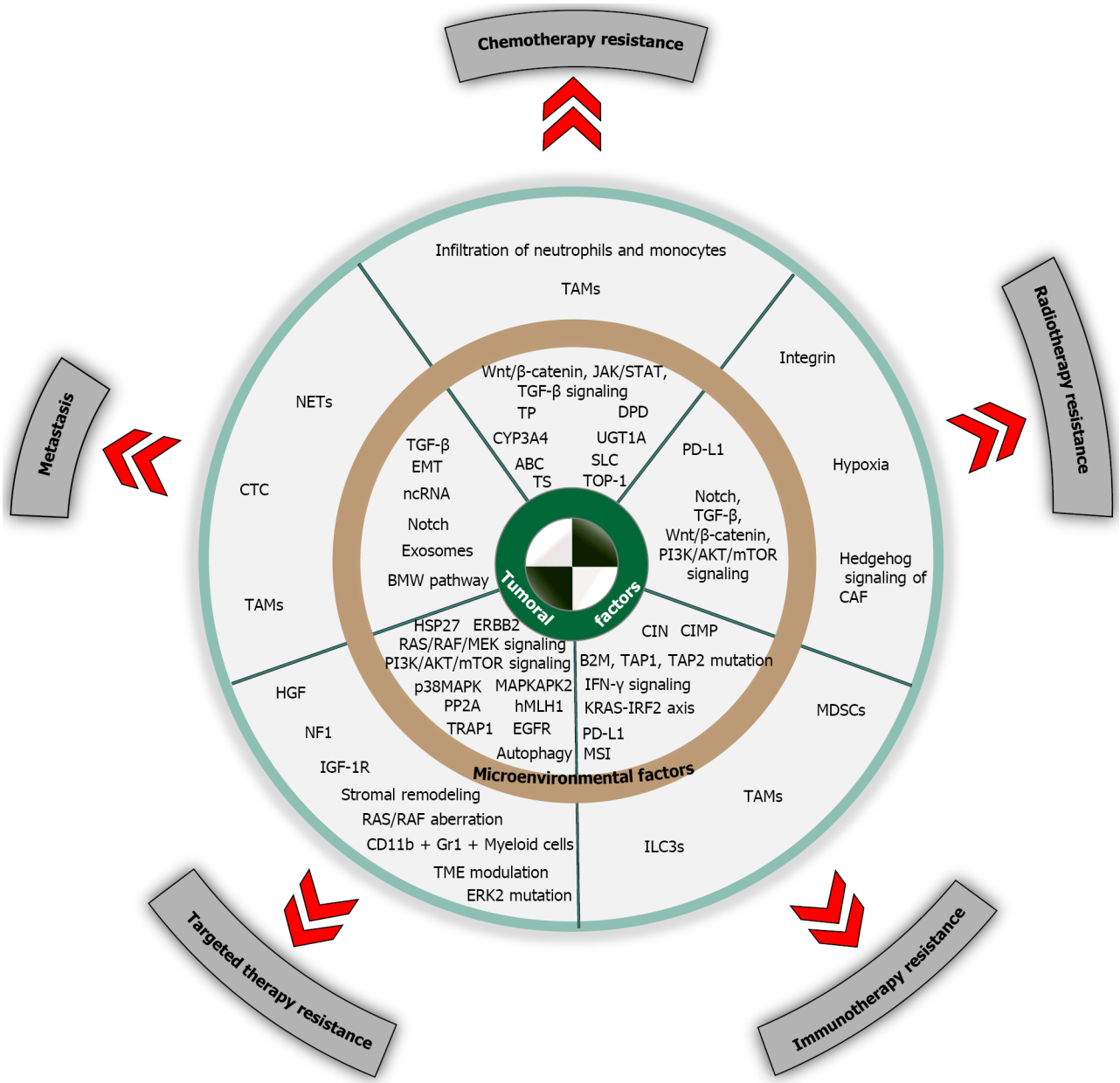
Fluorouracil, oxaliplatin, and irinotecan are the three main reagents used for CRC adjuvant chemotherapy, which exert antitumor effects through interaction with DNA. However, during the treatment, tumor cells adapt several mechanisms to evade the attack of the treatment agent. Generally, the tumoral mechanism of developing resistance can be categorized into 4 aspects: alterations in the pharmacokinetics and metabolism, transport process, and targets of chemotherapeutics as well as aberrant activation of oncogenic/bypass signaling (Figure 1).

In the case of pharmacokinetics and drug metabolism, reduced concentrations, decreased activation, and increased inactivation of drugs in tumor site, all contribute to the adaption of resistance. Efforts have been focused on investigating the importance of pharmacogenomics on intrinsic and acquired resistance in CRC. Polymorphism of genes encoding critical enzymes during drug absorption, distribution, metabolism and detoxication, has been identified which will facilitate targeted personalized therapy, such as UDP-glucuronosyltransferases (UGT1As) and glutathione-S-transferase (GSTs)[23]. The transformation of capecitabine (CAP) to 5-FU is essential for direct cytotoxicity, which is mediated by thymine phosphorylase (TP)[24]. According to a past report, tumor cells apply epigenetic alterations to downregulate the TP expression, which leads to the development of drug resistance to CAP, albeit it can be reversed by DNA methyltransferase[25]. Similarly, changes in the activity of the key enzymes of 5-FU and irinotecan were evaluated by CRC cells affecting drug sensitivity[8,9,26,27]. Moreover, the inactivation of drug metabolites facilitates the development of drug resistance by CRC cells. Considering that dihydropyrimidine dehydrogenase (DPD) catabolizes 5-FU into an inactive product, the investigation of CRC patients revealed that a high level of DPD expression was significantly related to the resistance to 5-FU[28]. Irinotecan and its active form SN38 can be converted into an inactive form by several enzymes, such as cytochrome P450 (CYP) 3A4 and uridine diphosphate glucuronosyltransferase 1A, and therapeutic strategies targeting these enzymes have been proposed to effectively reverse irinotecan resistance[29,30].

Changes in drug transport are another important factor for drug resistance. Membrane transport pumps can transport chemotherapeutic drugs or molecular targeted drugs out of the cells, thereby reducing the intracellular concentrations and leading to the development of multidrug resistance (MDR). Membrane transporters mainly include 2 families: ATP binding cassette (ABC) and solute carrier transporters. ABC transporters are the most famous and the main membrane transporter for MDR, executing an important role in CRC resistance to chemotherapy. P-glycoprotein (P-gp) is the first identified ABC superfamily member, and its upregulated expression has been implicated in the development of MDR leading to chemotherapy failure in CRC[31,32]. To date, several preclinical studies have demonstrated that natural inhibitors targeting ABCs or antisense oligonucleotide and ncRNAs aiming to downregulate the P-gp expression possess the therapeutic potential to reverse the resistance of CRC cells to oxaliplatin and 5-FU[33-36].

Alterations in the drug targets play a role in mediating the generation of drug resistance. Thymidylate synthase (TS) is the main target of 5-FU in destroying DNA replication, and its expression plays a crucial role in the development of CRC resistance to 5-FU[28]. Efforts to interfere with 5-FU-mediated TS inhibition are expected to disrupt the development of drug resistance. Folic acid (FA) enhances the cytotoxicity of 5-FU through the inhibition of TS both *in vitro* and *in vivo*[37,38]. Topoisomerase 1 (TOP-1) is the main target of irinotecan, and its mutation or downregulation is responsible for CRC resistance, as reduced TOP-1 binding to DNA also contributes to irinotecan resistance[27,39]. Past evidence suggests that Y-box binding protein 1 (YB-1) can improve the sensitivity of irinotecan through its direct interaction with TOP-1 toward promoting intracellular TOP-1 activity [40].

As for oncogenic/bypass signaling, it has been demonstrated that the sustained activation of Wnt/β-catenin, JAK/STAT, and TGF-β contributes to drug resistance of CRC. Wnt/β-catenin signaling is a well-known evolutionarily conserved pathway endowing tumor cells with sustained growth and is



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Figure 1 Schematic illustration of mechanisms of colorectal cancer cell resistance to chemotherapy, radiotherapy, immunotherapy, targeted therapy, and metastasis. ABC: ATP binding cassette; B2M: β2-microglobulin; CAFs: Cancer associated fibroblasts; CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; CTC: Circulating tumor cell; CYP3A4: Cytochrome P450 3A4; DPD: Dihydropyrimidine dehydrogenase; EGFR: Epidermal growth factor receptor; EMT: Epithelial-mesenchymal transition; ERBB2: Erb-b2 receptor tyrosine kinase 2; ERK2: Extracellular regulated protein kinases; HGF: Hepatocyte growth factor; hMLH1: Human MutL homolog 1; HSP27: Heat shock protein 27; IGF-1R: Insulin-like growth factor 1 receptor; ILC3s: Group 3 innate lymphoid cells; MAPKAPK2: Mitogen-activated protein; MDSCs: Myeloid-derived suppressive cells; MSI: Microsatellite instability; NETs: Neutrophil extracellular traps; NF1: Neurofibromin 1; p38MAPK: p38 mitogen-activated protein kinase; PP2A: Protein phosphatase 2A; SLC: Solute carrier; TAP1: Transporter 1; TAP2: Transporter 2; TAMs: Tumor associated macrophages; TME: Tumor microenvironment; TOP-1: Topoisomerase 1; TP: Thymine phosphorylase; TRAP1: TNF receptor associated protein 1; TS: Thymidylate synthase; UGT1A: Uridine diphosphate glucuronosyltransferase 1A.

responsible for CRC resistance to chemotherapy[41]. The inhibition of the Wnt/β-catenin pathway can restore the CRC sensitivity to 5-FU, thus exhibiting potential therapeutic potentials[42]. JAK/STAT signaling has been reported to regulate cell survival, proliferation, apoptosis, and differentiation, which is associated with CRC cells resistant to chemotherapy and immunotherapy[43-45]. TGF-β signaling also participates in the development of CRC resistance to chemotherapy through the upregulation of mesenchymal maker expression and inducement of MEK/ERK activation[46,47]. Targeted therapy against TGF-β thus presents promising value toward reducing the growth of chemo-resistant CRC cells and facilitating tumor control[48]. Last but not least, PI3K/AKT/mTOR pathway is well-accepted to be involved in tumorigenesis and development of CRC, which also contributes a lot to development of chemotherapy resistance[49]. Recent study identified miRNAs responsible for 5-FU resistance of CRC

cell lines through regulating activity of PI3K/AKT pathway[50]. Moreover, PI3K/AKT/HIF-1α and AKT/GSK-3β/Snail signals also contribute to drug resistance *via* regulation of glycolysis and stem cell maintenance, respectively[51,52].

An increasing body of evidence has demonstrated crosstalk between tumor cells and the microenvironment, and the impact of the TME on chemotherapeutic efficacy suggests another mechanism for drug resistance. For instance, tumor-associated macrophages (TAMs) facilitate tumor growth, metastasis, and angiogenesis, while suppressing the anticancer immune response, regulating metabolism, and even influencing the microbiota[53]. Recently, multi-omics research utilized a large sample to reveal increased infiltration of neutrophils and monocytes in chemotherapy-resistant CRC tissues, which has been associated with poor outcomes[54]. Meanwhile, another research by Gupta *et al*[55] investigated metastatic CRC patients who received neoadjuvant chemotherapy. The results revealed that the TME was significantly altered after chemotherapy when neoadjuvant chemotherapy increased the levels of T cells, especially $CD8^+$ T cells. Furthermore, bioinformatic studies focused on the prediction of sensitivity to chemotherapy and other therapies analyzing features of the TME and other pathological conditions have provided some promising results[56,57].

Epithelial-mesenchymal transition (EMT) also represents another form of interaction between tumor and microenvironment, especially during development of chemoresistance. Most tumor cells with the capacity of chemoresistance show a strong mesenchymal phenotype. EMT driven by miRNAs, oncogenes, protein kinases, and other factors facilitates to generation of drug resistance. Upregulated expression of DPYD, TYMS, ERCC1, and GSTP1 was associated with EMT, which eventually leads to 5-FU and oxaliplatin resistance of CRC cells[58,59]. DSTYK, a novel protein kinase, also contributes to chemoresistance in CRC cells by activating TGF-β induced EMT, and knockout of DSTYK hinders tumor growth *in vivo*[60]. Recently, proven by Zhu *et al*[61], activation of SOX2-β-catenin/Beclin1/autophagy signaling generated promoting effects on EMT, stemness and chemoresistance in CRC.

In addition to EMT, extracellular vesicles (EVs) are considered as a major pathway for cell-cell communication *via* transfer of their cargo, which ranges from proteins, lipids, miRNAs, and circRNAs. Due to the diversity of EVs cargo, it plays essential regulatory role during chemoresistance[62]. So far, compelling evidence indicated miRNAs transferred by EVs can be utilized as predictive marker of chemoresistance, and miR-21 was identified as the most common EV-associated miRNA relating to chemoresistance in CRC[63]. It was indicated that CRC exosomes are able to activate Wnt/β-catenin pathway through promoting the stabilization and nuclear translocation of β-catenin leading to 5-FU and oxaliplatin resistance[64]. CircRNA-122 delivered by EVs originated from chemoresistant CRC cells, played robust promoting effect on glycolysis which reduced drug susceptibility in chemosensitive cells [65]. In addition to harboring regulatory proteins, miRNAs, and circRNAs, drug efflux pumps (such as P-gp 1) and even cytotoxic drugs can also be transported by EVs to sequester drugs and negate drug effect on tumor cells[66-69]. Moreover, EVs generated from TME are also responsible for generation of chemoresistance of CRC. In a pre-clinical research, cancer-associated fibroblast (CAF)-derived EVs showed remarkable inhibitory effect on anti-cancer activity of oxaliplatin[70].

Radiotherapy resistance

Radiotherapy is considered another important adjuvant treatment to treat CRC, especially for advanced rectal cancer (as recommended by the NCCN guideline). However, resistance to radiotherapy is prevalent, which can be attributed to two main factors: tumor heterogeneity and the complexity of TME. Tumor heterogeneity is the most important reason for radiotherapy resistance, as supported by the fact that the equal effect of radiotherapy is not achieved in all tumor subpopulations. Among them, CSCs have been implicated as the most responsible factor for resistance to several therapies including radiotherapy[71-73]. Resistance to radiotherapy of CSCs can be categorized as intrinsic and acquired resistances, the latter of which is the adaptive response caused by radiotherapy itself. Owing to the distinct quiescent state and the plasticity of CSCs, they can survive stress, chemotherapy, and radiotherapy, which is referred to as intrinsic resistance[74]. Past studies have demonstrated that the activation of several signaling pathways contributes to CSC radio resistance, which includes Wnt/β-catenin, Notch signaling, TGF-β, Hedgehog, and PI3K/AKT/mTOR signaling. Wnt signaling increases the active levels of β-catenin post-radiation, leading to the development of resistance, which plays a role in the dedifferentiation of CSCs by regulating the SOX2 expression[75-78]. The Notch pathway can be activated *via* radiation and initiation of gene transcription, leading to an increased proportion of cells in the S phase[78]. The Hedgehog pathway is involved in the reprogramming of CAFs and is also responsible for the production of a supportive condition for the maintenance of CSCs stemness[79]. In addition to the alterations of the signaling pathways, tumor cells, especially CSCs, take adaptions of apoptosis, cell cycle, and EMT to survive radiation[74]. The cell-cycle progression can be blocked or slowed by irradiation that induces the redistribution of cells in the S phase, thereby contributing to radio resistance[80]. Researchers have found that the EMT triggered by irradiation may be related to the intravascular behavior of tumor cells forming the circulating tumor cells (CTCs) that are deemed responsible for the recurrence and metastasis[81,82].

Currently, a plethora of evidence indicates the significance of TME involved in radio resistance of solid tumors[74,83,84]. Concerns regarding the TME on radioresistance are based on the observation of COMMA-D cells, which are rarely tumorigenic and present with several features of normal mammary

epithelial cells. In a mouse model, the implantation of unirradiated COMMA-D cells into the mammary fat pads of irradiated hosts eventually formed large tumors, indicating the pivotal role of the TME in the determination of the radiotherapy outcome[85]. Generally, the effects of radiotherapy on the TME can be categorized into 3 forms: Vasculature, stroma, and immune system[84]. Irradiation damages the vasculature within tumors by causing the dysfunction of endothelial cells. The main consequence of radiation is hypoxia induced by increased permeability and detachment of the endothelial cells from the basement membrane as well as apoptosis[86,87]. To date, hypoxia has been recognized as one of the most critical reasons for radio resistance of tumors, and several strategies have been devised to reverse this resistance by overcoming the hypoxic environment. Ritter *et al*[88] reported that exposure to acute and adaptation to chronic hypoxia alters the balance of Bcl-2 family proteins in favor of anti-apoptotic family members, thus attenuating the success of radiotherapy[88]. Moreover, exposure to hypoxia can activate oxygen-sensitive signaling cascades leading to metabolic adaptation and increasing cell death threshold, which accounts for a worse response to radiotherapy[89].

Regarding the impact on the stroma, radiotherapy induces significant activation of CAFs. The upregulation of integrin by CAFs participates in radio resistance through the interactions among cancer cells, stroma cells, and the environment[90,91]. Finally, the immune effect of radiotherapy has been well studied considering that induction of immunogenic cell death (ICD) after irradiation can, in turn, induce anticancer response through the release of neoantigens[92]. The resultant change in inflammation can be caused by the alterations in the cytokine signaling pathways in the TME under hypoxia-created irradiation, which recruits both immunostimulatory and immunosuppressive cells to locate the tumor site[93,94]. Moreover, the exhaustion of $CD8^+$ T-cell induced by radiotherapy is another radio-resistant mechanism that is supported by the upregulation of PD-L1 on tumor cells after radiation therapy[95-97]. In such a case, the final immunomodulation of radiation therapy can be stimulatory or suppressive, which offers therapeutic challenges to confer the synergistic potential of radiation therapy combined with immunotherapy[84].

Immunotherapy resistance

Immunotherapy has long been recognized as an effective modality to treat multiple malignancies, albeit its efficacies on CRC remain confined to only a few subtypes. Generally, resistance to immunotherapy can be attributed to tumoral and microenvironmental factors. With regard to the tumor factors, it is well-acknowledged that CRCs with microsatellite stability is characterized by considerably low tumor mutation burden (TMB) and less immune infiltration when compared with CRCs exhibiting MSI. FDA-approved immunotherapy suggested certain outcomes in CRC patients with MSI metastatic disease[98,99]. In addition to MSI, two other pathways have been reported to be responsible for genomic instability, including chromosomal instability (CIN) and the CpG island methylator phenotype, whose identification can facilitate the stratification of immunotherapy-sensitive patients. In theory, the response to ICI is expected in all MSI tumors; however, a certain proportion of patients continues to experience progression due to several evading mechanisms in real clinical practice. Whether intrinsic or adaptive, the specific evading mechanism is not fully understood at present, although changes in some of the signaling pathways and gene expression have been reported[37,100,101].

The main mechanism concerns impaired antigen presentation or altered expression of HLA complex. Mutations of B2M [β2-microglobulin (B2M), or HLA class I heavy chain] in CRC have been reported to be significantly associated with the MSI phenotype but not MSS tumors[102,103]. Moreover, CRC patients with an MSI phenotype and harboring B2M mutations revealed resistance to anti-PD-1 mAb treatment[104]. B2M-mutant MSI tumors showed higher infiltration of PD-1+ T cells relative to B2M-wild-type MSI tumors, indicating the loss of HLA-I expression mediated by B2M mutation[105]. Moreover, the mutations of genes involved in the antigen presentation process, such as TAP1 and TAP2, have been related to the number of TILs in a tumor[106]. Other factors, including mutations in the HLA peptide-binding area, HLA class I transactivator NLR5, and RFX5, have also been cited as responsible for the altered function or expression of HLA that results in the development of immunotherapeutic resistance[101,106,107]. As for the signaling pathway, IFN-γ signaling plays an essential role in immunotherapy. The mutations of *JAK1* and *JAK2*-the downstream kinases of IFN-γ signaling-are associated with an inherent resistant to anti-PD-1 mAb treatment of CRC[45]. Recently, the KRAS-IRF2 axis was illustrated to be responsible for the direct regulate of the CXCL3 expression, which bound to CXCR2 on the surface of myeloid-derived suppressive cells (MDSCs) and promotes the migration of MDSCs to TME, resulting in the development of immunotherapeutic resistance[108].

On the other hand, TME is significantly related to resistance to immunotherapy. Most importantly, the density, function, and site of immune cells' infiltration in tumors are critical to the efficacy of the applied immunotherapy[109,110]. In such a situation, immunoscore is recommended for the evaluation of the immune contexture within a tumor. A score of 0-4 indicates a significant association with the prognosis of CRC patients[111]. Specifically, the expression of PD-L1 has been associated with a higher immunoscore[112,113]. Furthermore, researchers have suggested a novel CRC molecular subtyping system, and four consensus molecular subtypes (CMS) have fully illustrated CRC with distinct molecular and immune features[114]. According to the CMS subtyping, immunotherapeutic efficacy can be successfully predicted by different TMB and immunoscore, which is believed to be superior to those of conventional UICC-TNM staging[106,114-118]. Among the infiltrated immune cells, MDSCs and

TAMs, which are referred to as immunosuppressive myeloid cells, are the major suppressive factors that downregulate the antitumor effect of $CD8^+$T cells. Recently, researchers revealed a novel mechanism responsible for the regulation of MDSCs function through prostaglandin E2 receptor 4 (EP4), and they developed a selective antagonist TP-16 as a potential target for the enhancement of immunotherapy for CRC[119]. Furthermore, the microbiota has also been implicated in the development of immunotherapeutic resistance of CRC[120]. Group 3 innate lymphoid cells (ILC3s) was recently reported to be associated with the alterations in microbiota in human along with the indications of dysfunctional adaptive immunity and immunotherapy resistance[121].

Additionally, many studies have linked tumor EMT phenotype with immunosuppression status through regulating PD-L1 expression, which lead to resistance to therapies. Evidence from a former study has revealed the link between EMT and $CD8^+$T cell immunosuppression. MiR-200, a cell-autonomous suppressor of EMT, regulates expression of PD-L1, but suppressed by EMT activator ZEB1 [122]. Snail, a transcription factor of EMT, was shown to promote tumor growth *in vivo* and confer resistance against T cell-mediated lysis through the induction of autophagy[123,124].

Targeted therapy resistance

In addition to chemotherapy, radiotherapy, and immunotherapy, specific antibodies and small inhibitors therapy targeting cancer-associated receptors or signaling pathways represent another antitumor strategy toward control CRC. There are four main types of targeted therapy applicable in treating metastatic CRC: Monoclonal antibodies targeting EGFR, inhibitors of VEGF, small chemicals targeting intracellular kinases of several signaling pathways, and a recently approved small molecular BRAF inhibitor[125]. Among these, anti-EGFR therapy is widely used to manage KRAS wild-type CRC, including cetuximab and panizumab, the resistance to which will be mainly illustrated in this subsection. Reportedly, anti-EGFR therapy has been effective in 50% of CRC patients showing KRAS wild-type, but only 6% in those showing KRAS-mutant tumor[126,127]. In a clinical setting, patients usually develop resistance to anti-EGFR therapy after 3-12 mo of initiating the treatment[128,129]. Furthermore, it has been estimated that 80% of mCRC patients develop resistance to anti-EGFR regimens[130].

The mechanism of resistance to anti-EGFR therapy can be categorized into 2 types: Tumoral and environmental. Alterations of the EGFR ligands and EGFR significantly contribute to treatment resistance. The mutation of the EGFR kinase domain (V843 I) and the methylation of R198/R200 have been correlated with disease progression in patients receiving cetuximab[131]. Sequence changes in the extracellular domains of EGFR have also been implicated in conferring resistance to anti-EGFR treatment through the prevention of mAb binding[132-134]. The activation of compensatory feedback loop signaling, including RAS/RAF/MEK and PI3K/AKT/mTOR pathway, is also known to contribute to drug resistance[135]. Mutations, amplification, and the loss of genes, as well as aberrant phosphorylation of these pathways, are all important factors in the primary and secondary resistances to anti-EGFR therapy[136,137]. For instance, mutation of BRAF has been identified as the major marker of poor prognosis of mCRC after anti-EGFR treatment[138-140]. As a dominant downstream factor of EGFR signaling, mutations and abnormal activation of the PI3K/AKT/mTOR pathway is responsible for resistance to anti-EGFR[136,141,142]. Genomic stability is not only related to immunotherapy efficacy but also the development of anti-EGFR resistance. Moreover, it has been reported that hMLH1 deficiency generated resistance to cetuximab through the increased expression of ERBB2 and downstream PI3K/AKT signaling[143]. On the other hand, EGFR-targeted treatment has been reported to increase the levels of immune cells' infiltration and PD-L1 expression, suggesting the potential of treatment combination with immunotherapy to combat the resistance of CRC[135].

CSCs are considered the predominant reason for treatment resistance. After targeted therapy, CSCs aberrantly activate the substitute receptor tyrosine kinase, leading to the development of resistance to anti-EGFR therapy[144]. Moreover, they activate anti-apoptotic signaling to generate resistance to bevacizumab, such as PP2A, p38MAPK, MAPKAPK2, and Hsp27[145,146]. During an antitumor treatment, cancer cells takeover metabolic remodeling to sustain survival, which contributes to the development of resistance to targeted therapy. It has been reported that elevated levels of glycolysis mediated by TRAP1 is related to resistance to anti-EGFR therapy and that fatty acid metabolism demonstrated certain anti-apoptotic effect during the cetuximab treatment[147,148]. Furthermore, autophagy is responsible for developing resistance to the anti-EGFR regiment[149,150].

In terms of the environmental factors, TME plasticity plays a significant role in modulating resistance to targeted therapy[149]. The dysfunction of immune cells, altered infiltration of CAFs, and angiogenesis all have an impact on the efficacy of anti-EGFR treatment[151]. Hepatocyte growth factor (HGF) secreted by CAFs binds MET receptor and activates MAPK and AKT signaling, thereby inducing resistance of CRC cells to cetuximab[144]. Moreover, Woolston *et al*[129] applied genomic and transcriptomic analyses to evaluate primary and secondary resistances of CRC during anti-EGFR treatment and revealed that NF1 and non-canonical RAS/RAF aberrations are associated with primary resistance. Importantly, stromal remodeling mediated by CAFs has been indicated as a non-genetic mechanism of cetuximab resistance[152]. In addition, treatment-induced senescence is believed to exert a pro-tumorigenic effect, leading to recurrence and progression, which can be attributed to the secretion of several factors and genetic mutations[153,154].

To compensate for the prevalent resistance to anti-EGFR regimens, the combined application of other targeted therapies has been proposed and tested in preclinical and clinical studies. Although several agent-targeted RAS mutations have been approved in the treatment of CRC[155-157], the resultant response is not sufficiently long for most patients, and the underlying mechanism included the upregulation of some compensatory signaling events such as the TME modulation and recruitment of CD11b+ Gr1+ myeloid cells[158]. Anti-IGF-1R treatment has been recognized as a targeted strategy in the treatment of mCRC, the resistance to which has also been encountered in clinical studies. The underlying mechanism can be attributed to the nuclear location of IGF-1R during the treatment, which leads to the development of resistance to anti-IGF-1R therapy as well as chemotherapy[159]. In a study of 47 human CRC cell lines, the authors observed a synergistic inhibition effect with the combination of MEK and PI3K inhibitors, although primary and secondary resistances were observed and the mutation of ERK2 was recommended as the driver of resistance to the mono- or combined regimens[160].

Since approved by FDA in 2004, Bevacizumab (Avastin) and other VEGF pathway inhibitors have been utilized for cancer therapy, which improved survival in most cancer patients but some still have limited or no benefit from them. The primary and acquired resistance can be attributed mainly to activation of alternative angiogenesis other than VEGF pathway, including angiopoietin (Ang2), bombina variegate (Bv8), FGF, *etc*[161,162]. Additionally, other factors also play important role in development of anti-VEGF therapy resistance. Inflammation was tested to affect responsiveness to anti-VEGF in a mouse model of chemical induced colitis, which revealed Bv8/PROK2 expressed by tumor-infiltrating neutrophils related to anti-VEGF resistance[163]. Specifically, patients with $CD177^{+}$ neutrophil infiltration in tumor tissues had poor survival than those with $CD177^{-}$ infiltrates[164]. Besides, HOXB9 and extracellular matrix (ECM) remodeling induced by anti-VEGF were responsible for development of anti-VEGF resistance[165,166]. Park *et al*[167] found that nearly all of the cetuximab-resistant colorectal cancer cells showed a higher EMT signature, showing increased EMT markers such as SNAI2.

Metastasis

Metastatic events are the major cause of CRC patients' death, with the liver being the most common site of metastasis[168-170]. The lung, distant lymphatic nodes, and peritoneum are the secondary metastatic sites of CRC[168]. In the 1880s, the classic concept of "seed (tumor cells) and soil (specific organs) was proposed by Paget, which illustrated the basic metastatic mechanism for tumors[171]. To date, several excellent reviews have detailed the specific intravasation and extravasation procedures of CRC cells during metastasis[172-176]. For instance, liver metastasis is recognized as multiple factors- and processes-involved complex biological procedures. First, a certain subtype of CRC cells is endowed with the invasion capability, and they migrate to the ECM through the EMT, followed by infiltration into the surrounding tissues, after which they enter and survive in circulation, eventually colonizing and forming a secondary tumor in distant organs *via* extravasation[172]. Moreover, a metastatic mouse model explored by Enquist *et al*[177] suggested that the direct spread of CRC cells through the blood to the liver is a possible dissemination route without any prior lymph-node involvement; this information has the potential to facilitate future drug development targeting metastasis[177,178].

As far as the intrinsic factors are concerned, several molecular mechanisms have been deemed responsible for CRC metastasis, including non-coding RNAs (ncRNAs), Notch, TGF-β, and exosomes [172]. Among these, TGF-β is widely accepted as the most important signaling pathway related to metastasis. It was recently reported that an increased level of TGF-β in the TME promoted T-cell exclusion and blocked the acquisition of the T_H1-effector phenotype, while the blockage of TGF-β signaling rendered CRC susceptible to immunotherapy[179]. To induce the EMT process, TGF-β downregulated the expression of E-cadherin and upregulated the Vimentin expression in CRCs in order to promote invasion and migration[180]. Consequently, TGF-β signaling may serve as a vulnerable factor of CRC metastasis that can be exploited for targeted therapy in a clinical setting.

In addition to the EMT process mentioned earlier, recent studies have indicated a cell death process-dependent pathway for metastasis[181]. Among the several reversible cell death forms (such as apoptosis, necroptosis, ferroptosis, pyroptosis, and NETosis), the Blebbishield emergency program is considered a prototype for cell death-driven metastatic pathway[176]. The Blebbishield metastatic-witch pathway is characterized by the formation of apoptotic blebbing that is reconstructed into blebbishields and, subsequently, a spheroid state. This fusion structure may release or generate single cancer cells with the ability of migration, genomic instability, immune evasion, and increased metastasis[176,182-185]. On the other hand, it is believed that tumor cells must overcome multiple environmental obstacles during metastasis, and metabolic rewiring is widely adapted to achieve successful colonization in distant organs[186]. The adaption of pyruvate, lactate, glutamine, and fatty acids metabolism all contribute a several invasion and migration stages, the circulating stage, and the final colonization step, which represents a potential target for metastasis prevention and treatment[186-189].

Beyond the intrinsic factors of altered signaling and metabolic adaption, extrinsic conditions also participate in the regulation of CRC metastasis. Recently, neutrophil extracellular traps (NETs) formed by decondensed chromatin filaments of neutrophils were implicated in cancer metastasis[174,190]. Clinically, the increased accumulation of neutrophils has been revealed in pre-metastatic organs[191, 192]. Moreover, immunostaining of the CRC tissues demonstrated the existence of NETs in primary and

related metastatic lymph nodes of CRC[193]. Through *in vivo* and *in vitro* assays, NETs were found distributed at the primary site and over the tumor boundary of CRC, whose levels were increased in CRC patients[193-196]. The relevance between NETs and CRC progression and prognosis was illustrated with the assistance of a mouse model and an observational study[197]. Functionally, NETs promote proliferation and the secondary metastasis of CRC, as well as prepare for the adhesion of CTC to the liver or the lung epithelial cells[198-202]. It was also reported that the disruption of NET formation by DNase facilitates the control of CRC metastasis[194,196,199,203,204]. As a pivotal component of the TME, immune cells were found to be involved in CRC metastasis. TAMs have been reported to induce the EMT process for the promotion of the metastatic capability of CRC and CTC-mediated metastasis, which subsequently lead to the production of CCL2 for enhancing macrophage recruitment[205].

APPLICATION OF NANOTECHNOLOGY IN CONQUERING THERAPEUTIC RESISTANCE AND METASTASIS

As mentioned earlier, 2 main reasons result in treatment resistance and metastasis of CRC, namely tumor and environmental factors. Despite the advancements in cancer treatment and the development and design of several drugs or methods to target metastatic sites or reverse drug resistance, only a few such approaches have achieved satisfactory clinical outcomes. The rapid advancements in nanotechnology have provided new methods and options for the treatment of cancer with unique advantages, such as an excellent carrying capacity, better permeability, and the realization of multidisciplinary therapy[206]. In terms of nano delivery methods, compelling reviews have fully illustrated the diversity of nanomaterials currently utilized for cancer therapy, including organic materials, inorganic materials, polymers, and exosomes, *etc*. Exosomes, particularly, have been explored as a potential candidate in therapeutics delivery owing to their intrinsic targeting property, endogenous functionality, and ability to cooperate with a host defense mechanism. So far, exosome-based nanotechnology has achieved marked technological advances and exosome-based nanotheranostic platforms have thus been bloomed [207]. It has been demonstrated that exosome delivery led to higher drug accumulation in target cancer cells and improve molecule stability as well as blood circulation time, thus improving the efficiency of small molecule drugs[208]. Besides, exosomes secreted by different cell types contains a diversity of proteins which facilitates better recognition of targeted cells and reduce nonspecific distribution[209, 210]. In general, nanotechnology-based strategies have been developed to restore drug sensitivity and enhance the anti-tumor efficacy. The detailed advancement in accordance with the mechanism of therapeutic resistance and metastasis, will be discussed in detail in the following section.

Nanotechnology and surgery

Until date, surgery remains the preferred scheme of CRC and the only curative option for malignant bowel obstruction in some cases. As surgical procedures facilitate thorough and timely removal of the primary cancer site, the possibility of disease cure is ensured in most cases. However, 2 major challenges remain, including precise identification of micro-metastatic lesions during the surgery and the generation of a suppressive immune microenvironment after the surgery. Taking advantage of fluorescence imaging technology, researchers have developed novel intraoperative imaging methods that can specifically detect the tumor edge and micro-metastatic sites. For instance, Li *et al*[211] designed a fluorescent probe activated by a CD13/aminopeptidase N (APN), YH-APN, which demonstrated superior tumor-to-normal (T/N) tissue ratios under different conditions. Moreover, owing to the superior ability to distinguish metastatic lesions, even those as small as 1 mm in diameter, image-guided resection, and imaging of metastatic cancer were successfully achieved by spraying YH-APN. Recently, Wang *et al*[212] also used an *in situ* spraying method that enabled precise distinguishing of the tumor edge under the guidance of fluorescent probes. During surgery, the use of real-time fluorescent imaging to detect β-glucuronidase (GLU) activity was achieved by $DCDNO_2$, and the outlines of the tumor were visualized, which guided precise resection of liver cancer.

Regarding the suppressive immune microenvironment, Ji *et al*[20] designed a biopolymer nanogel implant that can be placed in the resection cavity of CRC (Figure 2). Owing to its good tissue adhesion ability and biodegradability, the implant remained in the surgical site and the loaded drugs, including antibody and resiquimod (R848), were released gradually. In the implant group, the residual tumors were eradicated after surgery, which helped inhibit distal tumor metastasis and induced immune-memory effects resisting the re-challenge experiment. Recently, Si *et al*[21] further improved a nanogel implant to an *in-situ*-sprayed gel delivering anti-OX40 antibody, which demonstrated the promising therapeutic potential of CRC. This improved nanogel modulated the suppressive immune microenvironment by inhibiting the activity of cyclo-oxygenase-2 (COX-2); as a result, the loaded aOX40 was gradually released for over 20 days, serving as an immune agonistic antibody. The treated mice displayed resistance to the re-challenge with M38 cells and a remarkable inhibition of the growth of abdominal metastatic tumors[21].

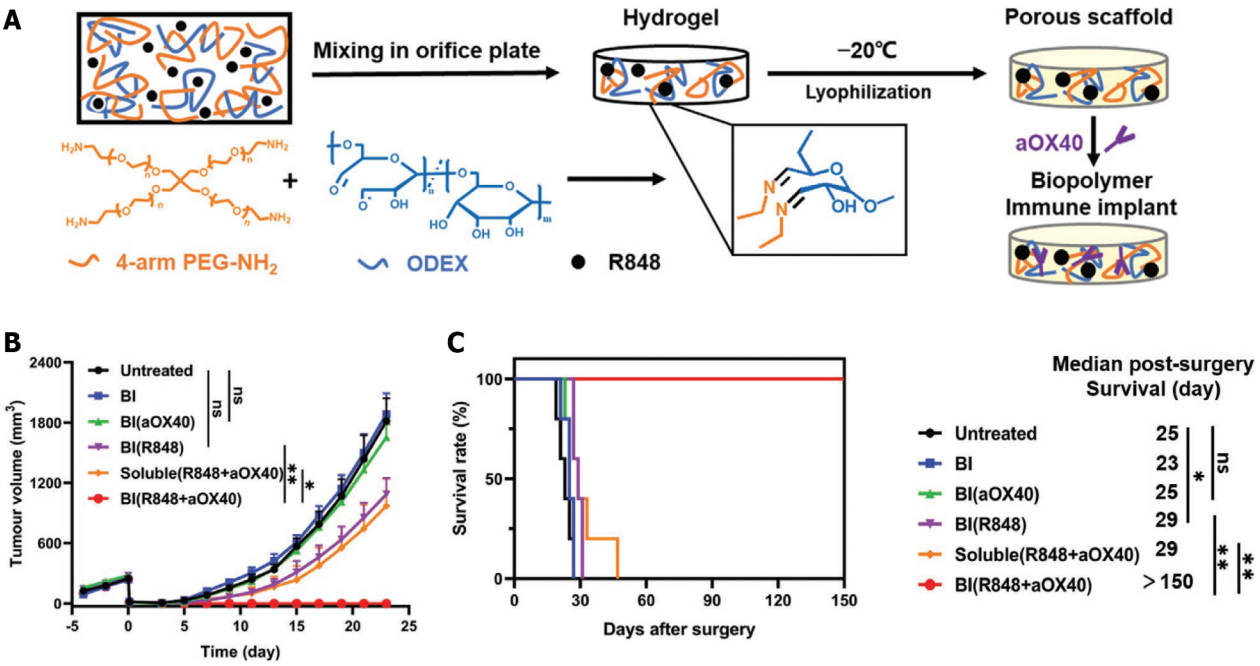


Figure 2 Intraoperative implant of biopolymer exhibited therapeutic effect on colorectal cancer growth. A: Schematic illustration of the implant preparation process; B: Immunoimplant inhibited tumor growth post-surgery; C: Immunoimplant showed survival benefit after tumor re-challenge. Citation: Ji G, Zhang Y, Si X, Yao H, Ma S, Xu Y, Zhao J, Ma C, He C, Tang Z, Fang X, Song W, Chen X. Biopolymer Immune Implants' Sequential Activation of Innate and Adaptive Immunity for Colorectal Cancer Postoperative Immunotherapy. *Adv Mater* 2021; 33: e2004559[20]. Copyright ©The Author(s) 2021. Published by Wiley-VCH GmbH Publications. The authors have obtained the permission for figure using from the Wiley-VCH GmbH Publications (Supplementary material).

Nanotechnology and chemotherapy

The therapeutic strategy of nanomaterials-derived drug delivery system toward overcoming chemotherapy resistance can be generally summarized into 4 aspects: (1) Increasing the drug concentration, accessibility, and permeability; (2) targeting the key genes of drug metabolism; (3) targeting the underlying efflux mechanism of the drug; and (4) the delivery of phytochemicals as an alternative.

The commonly applied strategy for overcoming drug resistance is to increase the local drug concentration. Improved cytotoxicity to human CRC cell lines was achieved through the direct delivery of DOX and 5-FU *via* oleic acid, which reduced the IC_{50} values relative to that with the free-drug forms[206,213]. The codelivery of chemotherapy and targeted therapy by nanomaterials also demonstrated an improved synergistic effect on the CRC cells. Recently, nanoemulsion loaded with paclitaxel (PTX) and BEZ235-a PI3K/AKT/mTOR pathway inhibitor-displayed a greater antitumor effect with remarkably lower IC_{50} when compared with free PTX. The combined therapy with nanoemulsion inhibited the growth of drug-resistant CRC cells either in culture or *in vivo* (Figure 3)[214]. Furthermore, theranostics mediated by magnetic resonance imaging (MRI) was applied to combat drug resistance through precise diagnosis and targeted therapy[215]. The application of the pH-responsive polymer PEALCa nanomicelles loaded with PTX and SPIO avoided the uptake by RES, entered human CRC cells, reached the lysosomes, and released PTX *in vitro*. The drug uptake efficiency could be visualized by MRI, which exhibited a relatively more efficient antitumor effect[216]. Further attempts took advantage of the imaging characteristics of materials to deliver drugs, realize real-time imaging of the drug uptake, as well as to control tumors simultaneously[217-219]. The decreased permeability of a drug is another reason for the development of resistance to it. For instance, Bergers *et al*[186] designed a nanogel loading 5-FU nucleoside floxuridine or other chemical agents, like gemcitabine. Their conjugated nanogel-chemotherapy system exhibited an improved anticancer effect on drug-resistant Caco-2 cells, with enhanced permeability across the gastrointestinal barrier.

Targeting mutations or the altered expression of genes involved in drug metabolism also serves as a promising option to resolve the issue of drug resistance. The upregulated expression of DPD (DPYD) has been implicated in the development of 5-FU resistance, as it catalyzes 5-FU into an inactive metabolite[220,221]. With the intension to reverse the resistance to 5-FU of SW480 cells, Chen *et al*[189] designed hollow mesoporous silica nanoparticles (HMSN) to deliver 5-FU. When grafted with EGF targeting to EGFR-overexpressed SW480/ADR cells, elevated intracellular 5-FU was recorded, which induced apoptosis and cell-cycle arrest, along with the downregulation of DPYD. In addition, restorage of sensitivity to 5-FU was achieved with the use of multi-walled carbon nanotubes and several other nano-based delivery systems with the capability to boost the antitumor efficacy of chemotherapeutics [222,223].

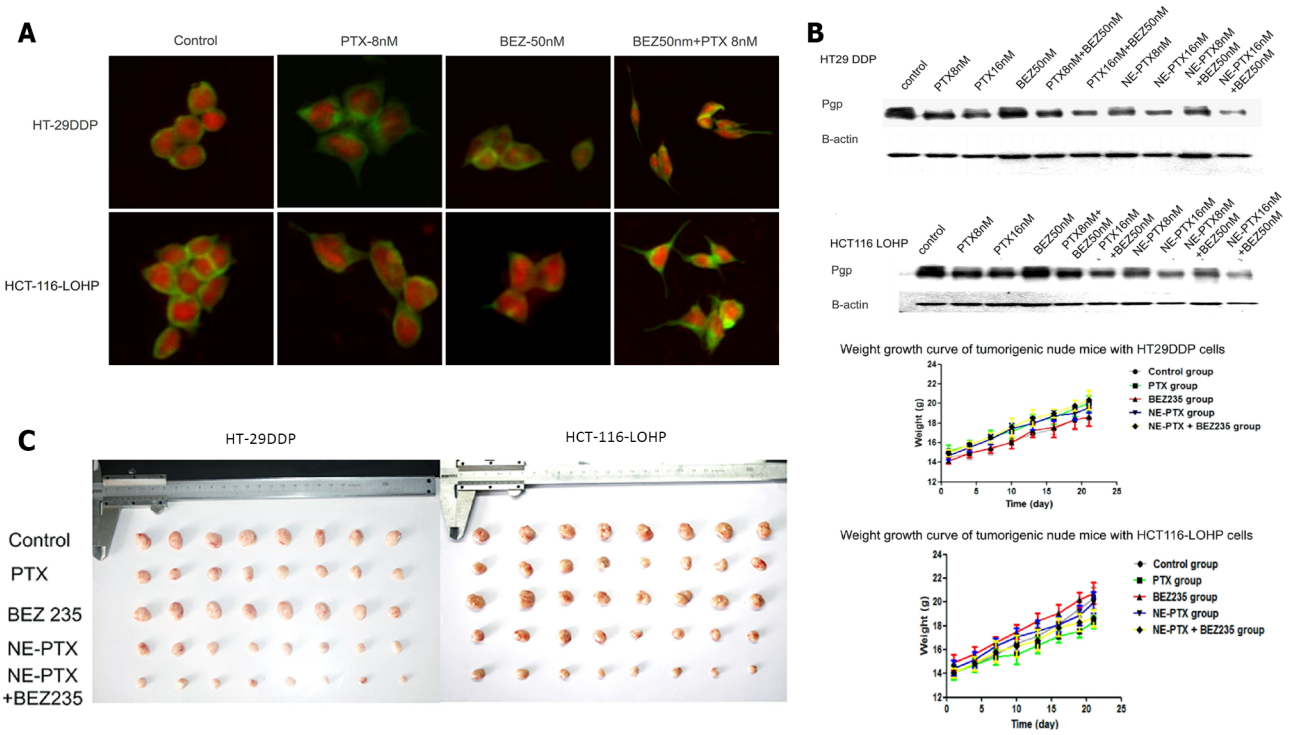


Figure 3 Nanoemulsioned Paclitaxel and BEZ235 reduced multidrug resistance of colorectal cancer. A: Morphological changes of cells treated with nanoemulsioned Paclitaxel and BEZ235; B: Downregulated expression of P-gp after NE-PTX and BEZ235 treatment; C: Synergistic inhibition of CRC growth *in vivo*. Citation: Hu Y, Zhang K, Zhu X, Zheng X, Wang C, Niu X, Jiang T, Ji X, Zhao W, Pang L, Qi Y, Li F, Li L, Xu Z, Gu W, Zou H. Synergistic Inhibition of Drug-Resistant Colon Cancer Growth with PI3K/mTOR Dual Inhibitor BEZ235 and Nano-Emulsioned Paclitaxel *via* Reducing Multidrug Resistance and Promoting Apoptosis. *Int J Nanomedicine* 2021; 16: 2173-2186[214]. Copyright ©The Author(s) 2021. Published by Dove Medical Press Ltd. The authors have obtained the permission for figure using from the Dove Medical Press Ltd. (Supplementary material).

Improvement in the biodistribution and pharmacokinetics of cytotoxic drugs is possible through the adoption of an environmentally responsive nanomaterial-derived delivery system. As reported by Udofot *et al*[224], pH-sensitive liposomal nanoparticles entrapping 5-FU modified with an anti-EGFR antibody (pHLNps-5-FU) exhibited prolonged plasma circulation, while accumulated exposure of pHLNps-5-FU in a tumor site exhibited enhanced anticancer effect relative to that with a free drug[224]. Au nanoparticles (AuNPs) are used to increase the 5-FU efficacy and decrease the related side effects; moreover, AuNPs-based nanoformulation has been reported to enhance the cytotoxicity of 5-FU to CRC cells[225]. Targeting the efflux mechanism to increase the intracellular accumulation of drugs serves as another effective approach to restoring drug sensitivity. For example, NPs designed with a PLGA core and an HA polymer shell loading 5-FU and L3-encoding cDNA (plasmid, pL3) were reported to sensitize CRC cells to 5-FU, as pL3 protein interfered with drug efflux *via* the P-gp regulation[226].

In addition to the abovementioned efforts, alternative therapies have achieved considerable outcomes with the use of naturally available phytochemicals, including curcumin, genistein, lycopene, and resveratrol, among others[227]. Thanks to nanotechnology, novel nanoformulations delivering phytochemicals have realized more efficiency and reduced side effects, which compensate for poor absorption, low solubility, and rapid metabolization of these natural drugs. Liposomes have been applied to enhance the uptake rate of curcumin, and its IC_{50} is lower than that of oxaliplatin, which can reduce the expression of angiogenic factors, increase apoptosis, inhibit angiogenesis, and prevent CRC progression[228]. The simultaneous encapsulation of curcumin and DOX in liposomes have been reported to increase drug accumulation at the CRC sites, exhibiting a higher inhibitory effect compared to that with free DOX[229]. Similarly, the establishment of an amphiphilic block copolymer pluronic/polycaprolactone to encapsulate curcumin demonstrated a better targeting effect on the tumor sites as well as restored the sensitivity of tumor cells to drugs[230]. Considering its abilities of chemosensitization, antioxidation, and anti-inflammation, resveratrol was loaded into gold nanoparticles (GNPs) after tagging with radionuclide technitium ^{99}m; it was rapidly taken up by CRC cells, displaying obvious cytotoxicity to tumor cells and excluding normal colon cells[231,232]. In support, other past studies have confirmed that the combination of resveratrol with 5-FU increases the sensitivity of CRC cells to 5-FU[233-236].

Hui *et al*[237] loaded oxaliplatin into exosomes encapsulating PGM5 antisense RNA 1 by electroporation. The results suggested a significant inhibition of both CRC cell proliferation *in vitro* and tumor growth *in vivo*[237]. Tran *et al*[238] encapsulated aspirin into exosomes derived from colorectal cancer cells and strongly enhanced cytotoxicity of aspirin to cancer cells. Interestingly, this cytotoxic effect was

more pronounced to parental cells of the exosomes, reminiscent of homing effect.

Nanotechnology and radiotherapy

Radiotherapeutic resistance is developed through multiple mechanisms, as illustrated in Section 2. Accordingly, strategies for treating radiotherapy resistance can be categorized into 2 aspects: Investigation of newer biomarkers or signatures to predict radiotherapy sensitivity and the modification of the TME, such as hypoxia. Due to the intrinsic resistance to radiotherapy, it is necessary to establish methods for the detection and identification of sensitive cases before initiating treatment. Recent studies have been able to investigate the genetic signatures and normalize clonogenic assays to predict the radiotherapy response in a more precise and effective manner[239,240]. On the other hand, the modulation of the TME by nanotechnology has demonstrated an improved radiotherapeutic efficacy owing to 3 advantages: An improvement of hypoxia by the loaded cargos, the radiosensitization capacity of some nanomaterials, and the combined application of multiple therapies by using a nanoplatform.

Hypoxia is believed to be one of the most prevailing mechanisms for the development of radioresistance. Methods suggested to restore the tumor oxygen supply before or during radiotherapy include erythrocyte transfusion, erythropoietin administration, and hyperbaric oxygen (HBO) treatment, among others. To date, compelling evidence has demonstrated that hypoxia can be successfully overcome with the use of relevant nanomaterials, as also summarized by several excellent reviews[241-243]. For instance, some simulated oxygen- or water-soluble components can be employed to improve the effect of radiotherapy. In one study, the bioreactive albumin MnO_2 nanoparticles synthesized by nanotechnology produced more oxygen in the hypoxic area, which was helpful to improve the radiotherapy effect[244]. Other strategies adopted so far include the delivery of exogeneous O_2 to the tumor site, *in situ* generations of O_2 and reduced consumption of O_2 in a tumor. Moreover, Tang *et al*[245] developed a nanoeconomizer to overcome radio resistance by regulating the hypoxic environment within tumors. This system functions in two ways: First, it releases NO in the acidic microenvironment to protect endogenous oxygen supply and reduce oxygen consumption; second, the photothermal effect facilitates the clearing of the residual tumor lesions that are resistant to radiotherapy (Figure 4).

Currently, nanoparticles with high atomic numbers, including metal-based NPs, have drawn much interest as radiosensitizers[246-248]. The level of GNPs taken up by CAFs in the TME is higher than that of tumor cells and normal fibroblasts. Therefore, during radiotherapy, GNPs can increase the local radiation dose and reduce the toxic effect on normal tissues, which improves the overall radiotherapy effect. Bromma *et al*[249] compared GNPs uptake by CAFs and DNA damage foci in CAFs to that in normal fibroblasts and HeLa cells and found that GNPs act as an effective tool in cancer radiotherapy to improve the overall efficiency. GNPs are commonly used as a radiosensitizer in *in vitro* experiments, albeit only a few have been used in clinical experiments due to their redox reactivity. Moreover, Guerra *et al*[18] designed an intercomparison of the respective sensitization capacities of GNP and iron oxide nanoparticles in human glioblastoma cells; they found different sensitization mechanisms, suggesting metal-based NPs as effective alternations to eliminate radioresistance.

Finally, through functionalization, the nanomaterials-derived system can be developed to realize disciplinary treatment simultaneously. The combined utilization of radiotherapy and immunotherapy exhibited great synergistic potential in the treatment of cancers. To overcome hypoxia and an immune suppressive microenvironment, Li *et al*[19] constructed a nanoprobe based on quantum dots that emitted in the near-infrared IIb window. By aggregation of nanoprobes in a tumor site, precision radiotherapy was realized with only a few side effects, improved ICD-augmented activation of dendritic cells, and induced robust T cell-mediated antitumor effect. Recently, Guan *et al*[16] engineered a nanoplatform combining radiotherapy and immunotherapy and discovered the potential to remodel the suppressive microenvironment after surgery. They constructed nanomedicine IPI549@HMP-targeted myeloid cells and catalyzed endogenous H_2O_2 to O_2, thereby reversing hypoxia-related radioresistance, which increased susceptibility to anti-PD-L1 therapy and eventually resisted tumor rechallenge.

Wang *et al*[250] developed exosomes from γδ-T cells and found that exosomes from γδ-T cells can specifically target the radioresistant $CD44^+$/high CSCs in nasopharyngeal carcinomas. Moreover, exosomes combined with radiotherapy had a higher therapeutic potency than radiotherapy monotherapy *in vitro* and *in vivo*. Wan et.al found that microRNA-34c-5p (miR-34c) could inhibit malignant behaviors in nasopharyngeal carcinomas. Exosomes overexpressing miR-34c significantly increased radiation-induced apoptosis in nasopharyngeal carcinomas[251].

Nanotechnology and gene therapy

Gene therapy represents a novel tool for conquering therapeutic resistance of CRC in a specific and targeted method, considering that genetic alterations are generally responsible for treatment failure. Strategies involving gene therapy include gene replacement, gene addition, gene expression modifications at the RNA level, and gene editing at the DNA level[252,253]. The combination of molecular biological technologies and nanomaterials has significantly promoted the development of gene therapy for CRC. Pigment epithelium-derived factor (PEDF) protein, a well-accepted tumor suppressor, has been reported to inhibit tumor proliferation and progression through its anti-angiogenesis and pro-apoptotic effects. Recently, Bao *et al*[254] designed a liposome-loading PEDF-DNA decorated with an

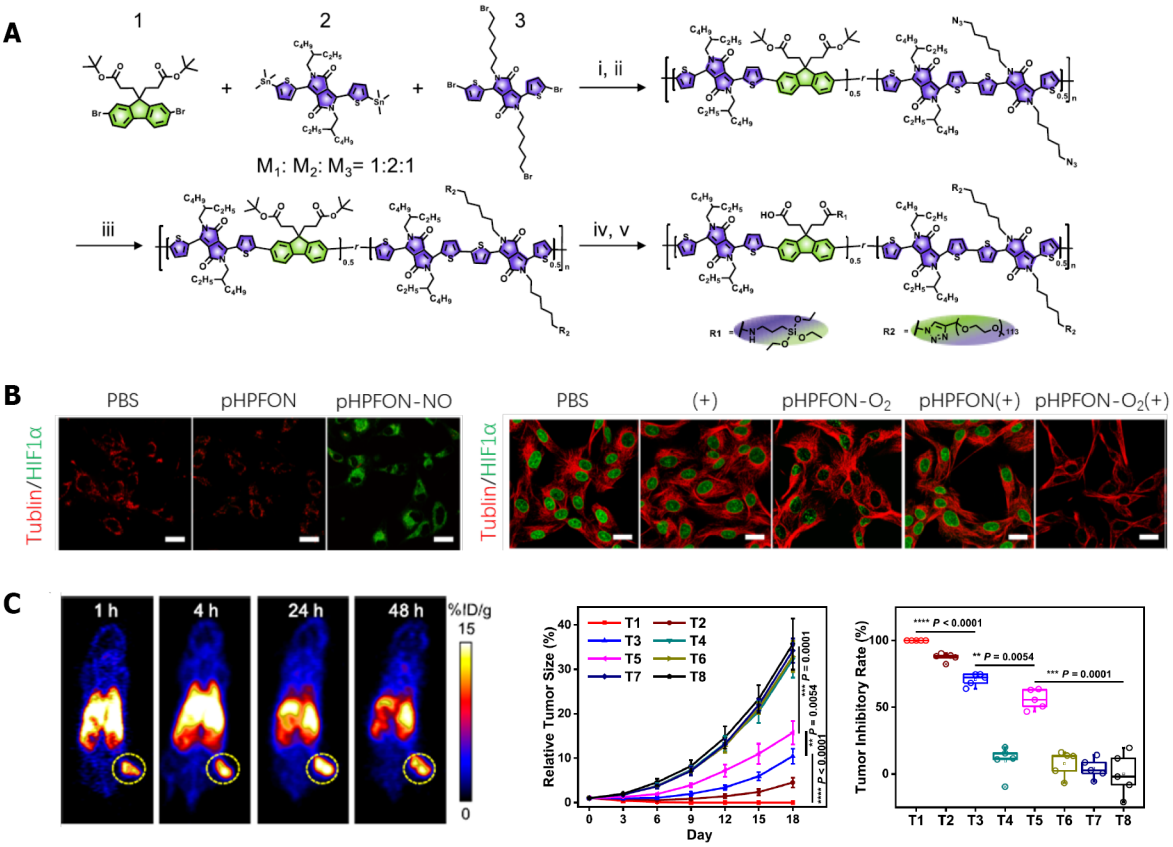


Figure 4 Organosilica-based O_2 nanoeconomizer improved radiotherapy efficacy. A: Design and synthesis of nanoeconomizer; B: pHPFON-NO reduced oxygen consumption (left), and broadened oxygenation (right); C: Multi-modal imaging by pHPFON-NO/O_2, and inhibited tumor growth after pHPFON-NO/O_2 administration. Citation: Tang W, Yang Z, He L, Deng L, Fathi P, Zhu S, Li L, Shen B, Wang Z, Jacobson O, Song J, Zou J, Hu P, Wang M, Mu J, Cheng Y, Ma Y, Tang L, Fan W, Chen X. A hybrid semiconducting organosilica-based O(2) nanoeconomizer for on-demand synergistic photothermally boosted radiotherapy. *Nat Commun* 2021; 12: 523[245]. Copyright ©The Author(s) 2021. Published by Springer Nature Ltd. The authors have obtained the permission for figure using from the Springer Nature Ltd. (Supplementary material).

iRGD peptide and demonstrated the reduced metastatic capability of CRC cells and the induction of apoptosis *in vitro*, as well as the inhibition of lung metastasis and prolonged survival of the animal model.

In addition to delivering DNAs encoding functional proteins, gene silencing therapy is commonly applied in CRC treatment. The methods adopted for gene silencing usually comprise the delivery of antisense oligonucleotide, miRNA, and siRNA, among which siRNA delivery is believed to offer more advantages[255-259]. Moreover, CRISPR/Cas9 technology has achieved a better targeting effect relative to siRNA therapy[260,261]. Recently, Zhao *et al*[262] constructed a cationic liposome-based nanoparticle for purpose of delivering miR-139-5p, decorated with epithelial cell adhesion molecule aptamer on the surface for the targeted treatment of CRC. With the use of cell lines and animal models, the constructed nanoparticle exhibited a satisfactory uptake and targeting ability, while demonstrating a tumor-growth suppressive effect.

On the other hand, siRNA delivery has achieved considerable outcomes through silencing of the key genes related to metastasis and MDR in order to reverse drug resistance of CRC cells[263,264]. To date, several nanocarriers have been developed to deliver siRNAs, which include liposome, PLGA, and RNA nanoparticles. Liposome-derived system is the most common platform for delivering siRNAs, which can protect siRNAs from degradation and kidney clearance while offering the advantages of convenient manufacturing and biodegradability[263]. However, the utilization of liposomes continues to be hindered by issues related to toxicity and release, and PLGA polymers were recently proposed as the preferred agents to realize prolonged release circumventing these obstacles while showcasing better biodegradability and lower toxicity[264-266]. Thanks to rapid development, nucleic acid nanotechnology has gained much attention with its distinct features. Particularly, RNA nanoparticles are widely being used to deliver multiple drugs, thanks to their low toxicity, negative potential, programmable features, and the ability to self-assemble and multi-valent capacity[267]. Rychahou *et al*[268] developed a multifunctional RNA nanoparticle targeting FA and achieved an excellent target effect to CRC metastatic cells mediated by FA-receptor mediated endocytosis, which demonstrated no accumulation in the normal tissues of the targeting organs such as the liver, lymph nodes, and lung. Moreover, the combination of siRNA delivery with RNA nanoparticles was recently applied in the treatment of lung

cancer, breast cancer, and prostate cancer, which effectively inhibited the Survivin expression and slowed the tumor growth *in vivo*[269].

Taking advantage of the abovementioned nanocarriers, siRNA therapy can target various genes or be combined with other treatments. Antigen-presentation cells (APCs) and KRAS are well-known oncogenes responsible for CRC development. Recently, Wan *et al*[270] designed a nanomedicine based on the CRISPR/Cas9 technology, which simultaneously targeted APC and KRAS mutation CRCs. A duplex CRISPR/Cas9 ribonucleoprotein was designed to deliver APC and KRAS sgRNA encapsulated in a phenylboronic dendrimer decorated with hyaluronic acid (HAPD). The resultant nano-complex was administered systematically to Balb/c nude mice, which displayed a synergistic targeting effect on APC and KRAS mutations along with a remarkable reduction in tumor growth. Moreover, the therapeutic capacity for metastasis was further illustrated by the establishment of the orthotopic CRC mouse model and the liver and lung metastasis models, whose survival rates were significantly prolonged (Figure 5)[270]. Intergrin-β1 is one of the key factors involved in CRC metastasis, which is upregulated in CRC cells resistant to regorafenib[271]. The delivery of siRNA-targeting intergrin-β1 to regorafenib resistant CRC cells *via* regorafenib/dimethyldioctadecylammonium bromide (DDAB)-methoxy poly (ethylene glycol) (mPEG)-poly-ε-caprolactone hybrid nanoparticles (HNPs) was found to downregulate the intergrin-β1 expression and induce apoptosis, thereby successfully enhancing the overall sensitivity to regorafenib[271]. P-gp is responsible for drug pump leading to decreased drug concentrations in the cells, which has been proposed as another target of siRNA therapy. The delivery of P-gp siRNA with lipofectamine achieved an efficient control of CRC cells[272]. In addition, the delivery of siRNA-silencing TGF family loaded in tripolyphosphate-decorated chitosan nanoparticles inhibited the formation of liver metastasis of CRCs *in vivo*[273]. The co-delivery of multiple siRNAs or combination with other treatments, such as chemotherapy, can thus be considered as an alternative strategy for the improvement of CRC control[274].

Li *et al*[275] suggested that miR-3940–5p is significantly downregulated in CRC. They loaded miR-3940–5p into exosomes derived from MSCs and transfected them into CRC cells. The results showed significant suppression of EMT and invasion *in vitro*, and inhibition of tumor growth and metastasis *in vivo*. Liu and coworkers evaluated the effect of MSC-derived exosomes transfected with miR-15a on CRC cells. The results indicated the suppression of CRC cell proliferation and tumorigenesis *in vitro* and *in vivo*[276].

Nanotechnology and immuno- and targeted therapies

Currently, it has been well-accepted that nanotechnology offers distinct advantages in improving the efficacy of cancer immunotherapy, which is widely used as the platform that enables the combined utility of immunotherapy with other treatments, along with the reprogramming of the tumor immunosuppressive microenvironment.

Firstly, it has long been established that targeted and immunotherapy can be combined with other anticancer treatments to achieve synergistic effects[6,277-279]. The introduction of nanotechnology to cancer immunotherapy, especially for CRC, is based on the hope of reversing the immunosuppressive microenvironment, inducting a robust immune response and long-term immune memory effect, as well as transforming non-responsive T cells into responsive T cells in the TME for MSS or pMMR CRC[280]. Ni *et al*[281], for instance, constructed a bi-adjuvant nanovaccine (banNV) that delivers the tumor antigen Adpgk, TLR7 agonist R848, and TLR9 agonist ODNs. The combined administration of banNV with anti-PD-1 antibody was found to suppress tumor growth in an orthotopic MC38 mouse model, prolonging the survival rate to 70% on day 48 of treatment. Furthermore, the use of nanomedicine co-delivering photothermal or photodynamic agents with immunotherapy treatment has bene reported to achieve a certain improvement in CRC control. Luo *et al*[282] took advantage of hollow gold nanoshell encapsulating anti-PD-1 peptide into biodegradable poly (D, L-lactic-co-glycolide) nanoparticles (AA@PN), as it overcomes the shortcomings of peptides that have a short half-life, poor stability, and off-target effects. On irradiation by a NIR laser, AA@PN generated photothermal ablation of tumors. In another study, through PD-1 blocking and photothermal ablation *via* NIR laser irradiation, perdurable and enhanced immune response was recorded in the CT26 mouse model that also prolonged the survival rate[282].

He *et al*[283] realized the potential of the combined application of chemotherapy, photodynamic therapy, and ICI therapy through the construction of a nanoscale coordination polymer core-shell nanoparticles-loading oxaliplatin in the core and photosensitizer in the shell. In CRC mouse models, the combined administration of nanoparticles and anti-PD-L1 antibody was found to promote the release of pro-inflammation cytokines and synergistically activate DC and $CD8^+$ T cells, which significantly controlled the local and distant tumor growth, with the primary tumors reducing in size by 2.9% in the PBS-treated group[283]. In addition, nanotechnology offers an optimal platform for radiotherapy and ICI therapy. A nanoscale metal-organic framework was established to enable radiotherapy and checkpoint blockade immunotherapy (indoleamine 2,3-dioxygenase, IDO). The abscopal responses were generated after irradiation, leading to an enhanced immune response in both CRC and breast cancer mouse models[284]. Recently, Guan *et al*[16] developed a novel therapeutic strategy targeting the suppressive microenvironment post-surgery by using the nanomedicine IPI549@HMP, which was composed of a PEGylated $HMnO_2$ (HMP) loaded with PI3-kinase γ (PI3kγ) inhibitor (IPI549). PI3Kγ,

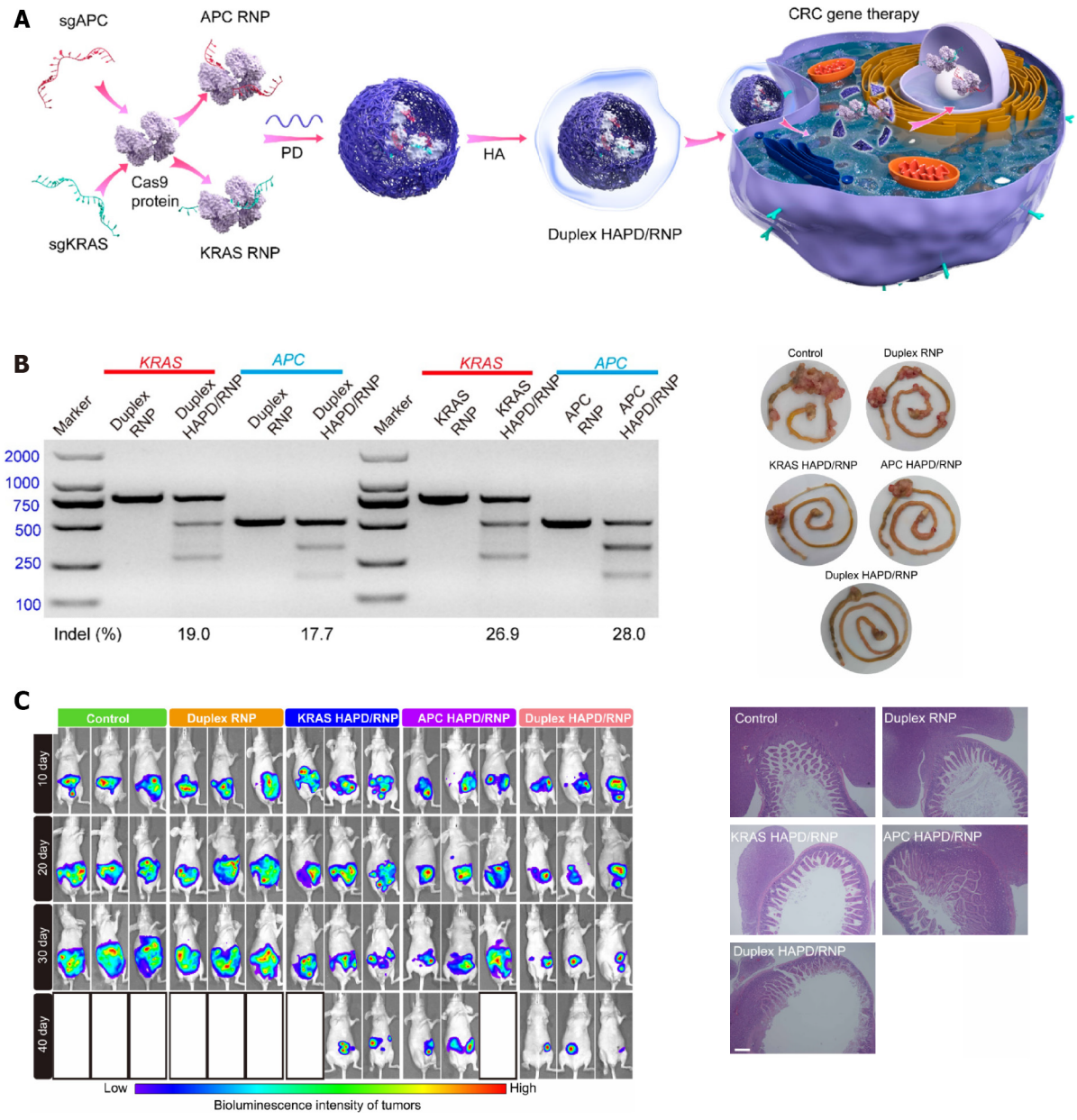


Figure 5 Duplex CRISPR/Cas9 ribonucleoprotein nanomedicine disrupted antigen-presentation cells and KRAS expression to treat colorectal cancer. A: Schematic illustration of preparation of nanoformulations; B: Disruption of antigen-presentation cells and KRAS expression after duplex HAPD/RNP treatment; C: *In vivo* luciferase expression of orthotopic CRC (left), and representative intestine images and H&E staining of intestine sections after nanomedicine treatment. Citation: Wan T, Pan Q, Liu C, Guo J, Li B, Yan X, Cheng Y, Ping Y. A Duplex CRISPR-Cas9 Ribonucleoprotein Nanomedicine for Colorectal Cancer Gene Therapy. *Nano Lett* 2021; 21: 9761-9771[270]. Copyright ©The Author(s) 2021. Reprinted permission from ACS Publications. The authors have obtained the permission for figure using from the ACS Publications (Supplementary material).

which is highly expressed on MDSCs, serves as the target molecule for the specific regulatory effect of IPI549@HMP on myeloid cells. Moreover, this nanomedicine catalyzed H_2O_2 into O_2 by remodeling the hypoxia environment after radiation. In this case, the combined use of IPI549@HMP with anti-PD-L1 antibody exhibited a synergistic effect on the elimination of CT26 cells and the suppression of distant metastasis, which significantly prolonged the survival of IPI549@HMP-treated mice up to 100 d[16] (Figure 6).

On the other hand, nanotechnology also contributes to the modulation of the immunosuppressive microenvironment, as illustrated by several excellent reviews[285-287]. Targeting APCs augments the antitumor effect by inducing a more extensive T-cell response. Zhu *et al*[288] designed self-assembled interwining DNA-RAN nanocapsules (iDR-NCs) that delivered CpG-coding DNA and STAT3-shRNA. The nanocapsules activated APCs by TLR9 signaling and relieved the inhibition of the CpG immunostimulatory effect by silencing STAT3. In an MC38 mouse model, the nanocapsules generated durable T cell responses, demonstrating potent synergistic immunostimulation effect[288]. Importantly, high levels of intratumoral lipopolysaccharide (LPS) are related to a poor response to anti-PD-L1 antibody treatment. Consequently, Song *et al*[289] used polymyxin B (PmB) to trap LPS inside the tumor. In

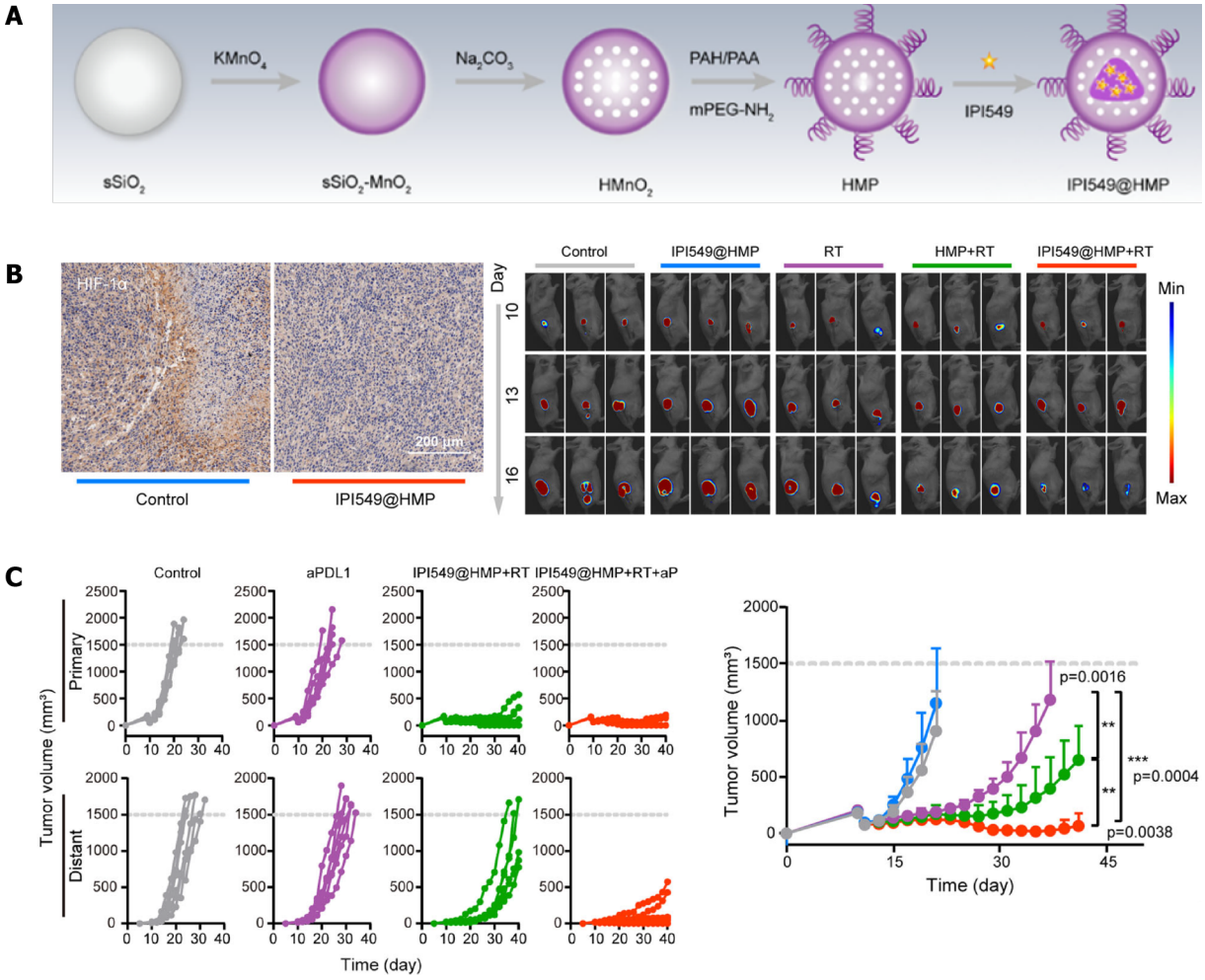


Figure 6 Nanomedicine targeted myeloid-derived suppressive cells exhibited synergistic inhibitory effect on colorectal cancer growth with radiotherapy and PD-L1 blockade. A: Schematic illustration of synthesis of HMP nanoparticles and drug loading; B: IPI549@HMP treatment improved hypoxia environment (left), and augmented radiotherapy efficacy; C: Kaplan-Meier survival curves and tumor growth curves of each groups. Citation: Guan X, Sun L, Shen Y, Jin F, Bo X, Zhu C, Han X, Li X, Chen Y, Xu H, Yue W. Nanoparticle-enhanced radiotherapy synergizes with PD-L1 blockade to limit post-surgical cancer recurrence and metastasis. *Nat Commun* 2022; 13: 2834[16]. Copyright ©The Author(s) 2022. Reprinted permission from Springer Nature Publications. The authors have obtained the permission for figure using from the Springer Nature Publications (Supplementary material).

combination with the TLR4 agonist TAK-242, a remodeled TME was achieved that allowed enhanced infiltration of the T cells in the tumor. The authors designed engineered LPS-targeting fusion protein, the coding sequence of which was loaded into a lipid-protamine-DNA (LPD) system. When combined with an anti-PD-L1 antibody, the nanosystem not only prevented liver metastasis of CT26-FL3 cells but also remarkably inhibited CT26-FL3 tumor cell growth in the liver[289].

In addition to its contribution to the development of immunotherapeutic agents mentioned earlier, nanotechnology serves as a promising tool to enhance the efficacy of targeted therapy. For instance, erlotinib, an orally active inhibitor of EGFR, renders resistance during the treatment of CRC, leading to treatment failure. In addition, Javadi *et al*[290] developed a methoxy polyethylene glycol poly caprolactone loading curcumin and erlotinib. The nanomicelles were administrated in combination and they decreased the expression of αvβ3 integrin while increasing the expression of PDK4 in resistant SW480 cells. Resistance to cetuximab, which is another commonly used anti-EGFR antibody, was found to be reversed with the use of AuNPs (designed as cetuximab-conjugated AuNPs by Hallal *et al*[291]) The application of AuNPs loaded with cetuximab demonstrated an improved endocytosis effect and an enhanced inhibition of the downstream signaling pathway of HT29 cells relative to those with the application of cetuximab alone, suggesting their potential in reversing resistance to EGFR treatment in CRC[291].

Naseri *et al*[292] demonstrated that exosomes derived from CSC-enriched colonospheres (CSCenr-EXOs) significantly increased the IL-12/IL-10 ratio in supernatants of mature DCs. Furthermore, CSCenr-EXOs-loaded DCs effectively promoted T-cell proliferation. Hosseini *et al*[293] loaded exosomes isolated from CT-26 cell line with miR-34a mimic and found that these exosomes could diminish gene expressions related to invasion, angiogenesis and immune evasion and induce cytotoxic T cells *in vivo*.

Nanotechnology-targeted TME

In addition to targeting the immune environment in TME, CAFs should be considered a critical therapeutic target for overcoming the resistance of CRCs. Recently, the functions and regulatory mechanisms of CAFs were reviewed by Kobayashi, who illustrated in detail the clinical implications of using CAFs as biomarkers and targets for controlling gastrointestinal cancer[294]. The authors further recommended 6 strategies to target CAFs: Elimination of CAFs by using CAR T cells or vaccination; reprogramming of cancer-promoting CAFs (pCAFs) into cancer-retarding CAFs (rCAFs); conjugation of antibodies with cytokines; the administration of modified MSCs; blockade of biochemical signaling; ECM-targeting therapy by depleting hyaluronic acid (HA) or the inhibition of lysyl oxidase. As far as CRC is concerned, there has been an increasing interest along with considerable achievement in the application of fibroblast activation protein-targeted vaccination that increases chemotherapy sensitivity, as well as reprograming of CAFs with vitamin D[295,296]. Recently, Ji *et al*[297] designed a novel cleavable amphiphilic peptide (CAP) targeting FAP-α that can load hydrophobic drugs and release the cargo in the presence of FAP-α. The designed drug-delivery strategy, called the CAP-NPs, demonstrated promising specific targeting effects of tumors and achieved therapeutic efficacy for several tumor models[297]. Collectively, much remains to be done to optimally utilize nanotechnology targeting CAFs toward reversing CRC therapeutic resistance and controlling distant metastasis.

CONCLUSION

Resistance to therapies and metastasis are the most critical reasons that ultimately result in treatment failure for CRC patients. The application of nanotechnology in the fields of surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy opens up broad prospects for overcoming drug resistance and controlling CRC metastasis owing to its unique targeted delivery, integration of multiple therapies, and synergistic therapeutic effects. For early-stage CRC, the modulation of the post-surgery microenvironment is worthy of investigation through the simultaneous implantation or spraying with nanomaterials-derived agents so as to improve the overall survival rate of the patients. As resistance to cytotoxic drugs is commonly encountered in a clinical setting, the codelivery of functionalized nanomaterials and natural phytochemicals is a novel alternative to restore the sensitivity of CRC cells. In radiotherapy, it is extremely promising to utilize the instinctive radiosensitivity of nanomaterials to reverse the hypoxic environment and combine it with immunotherapy to improve the overall efficacy of radiotherapy. In delivering gene therapy, nanotechnology offers distinct advantages that may be further studied to realize the specific antitumor effects. With the ongoing research on the immunotherapy mechanism behind CRC, it is necessary to work toward improving the immunomodulation effect of nanotechnology so as to convert non-reactive tumors into reactive tumors. In addition to targeting the immune cells in TME, CAFs can also be considered a promising target by nanotechnology to treat therapeutic resistance. Generally, treatment should not only target tumoral factors including genetic alterations, signal activation, or drug metabolites that lead to drug resistance but also pay attention to modulating the TME so as to eliminate the inhibitory factors and enhance powerful anticancer effects. Meanwhile, it is imperative to maintain a balance between the complexity of composition and structure and in the preparation and fabrication of nanoplatforms. Furthermore, better preclinical models are warranted to reflect the pathogenesis of CRC in order to achieve successful clinical translations of the emerging and established advancements in nanotechnology.

FOOTNOTES

Author contributions: Ren SN and Zhang ZY reviewed the literature and wrote the paper; Zhang ZY designed and draw the figures; Guo RJ, Wang DR, and Chen FF performed the collected data; Fang XD and Chen XB initiated the idea and revised the manuscript; all authors have read and approved the final manuscript.

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MINIREVIEWS

Interferon-lambda: New role in intestinal symptoms of COVID-19

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Abstract

The tremendous public health and economic impact of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a huge challenge globally. There is increasing evidence that SARS-CoV-2 induces intestinal infections. Type III interferon (IFN-λ) has an antiviral role in intestinal infection, with focused, long-lasting, and non-inflammatory characteristics. This review presents a summary of the structure of SARS-CoV-2, including its invasion and immune escape mechanisms. Emphasis was placed on the gastrointestinal impact of SARS-CoV-2, including changes to the intestinal microbiome, activation of immune cells, and inflammatory responses. We also describe the comprehensive functions of IFN-λ in anti-enteric SARS-CoV-2 infection, and discuss the potential application of IFN-λ as a therapeutic agent for COVID-19 with intestinal symptoms.

Key Words: COVID-19; SARS-CoV-2; Interferon-λ; Gastrointestinal infection; Mechanism; Anti-viral response

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Core Tip: The tremendous public health and economic impact of the coronavirus disease 2019 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a huge challenge for countries across the whole world. Increased evidences have revealed that SARS-CoV-2 also induced intestinal infection. Type III interferon (IFN), also called IFN-λ, plays an antiviral role in intestinal infection, and has focused, long-lasting, as well as non-inflammatory characteristics. In this review, we summarized the invasion and immune escape mechanisms of the SARS-CoV-2. Furthermore, we concerned about the gastrointestinal impact of the SARS-CoV-2 and the comprehensive role of IFN-λ, also called type III IFN, in anti-enteric SARS-CoV-2 infection.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) was first documented in December 2019, as an outbreak of acute community-acquired atypical pneumonia occurred in Wuhan, China, with unknown etiology. At that time, COVID-19 represented a newly emerged respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which subsequently became a pandemic[1].

Similar to the flu virus, SARS-CoV-2 spreads in small liquid particles from the mouth or nose of an infected person when they cough, sneeze, speak, sing, or breath. These particles range from larger respiratory droplets to smaller aerosols[2]. It is widely reported that coronavirus has the largest non-segmented genome (close to 30 kb) of all RNA virus. This unique genome size enhances the plasticity of CoVs, which alter through mutations and recombination[3]. The first SARS-CoV-2 variant, Alpha B.1.1.7, was reported in the United Kingdom in September 2020[4]. Increasing numbers of variants subsequently appeared, including Beta B.1.351, Gamma P.1, Delta B.1.617.2, and Omicron B.1.1.529[5]. By November 2021, Omicron B.1.1529 had become the current major SARS-CoV-2 variant globally[6]. Because of this strong plasticity in CoV genes, generating high genetic diversity and a high risk of cross transmission, specific treatments remain elusive.

As a systemic disease, SARS-CoV-2 can infect multiple organs, resulting in multiorgan dysfunction in COVID-19 patients. Organs that are infected by SARS-CoV-2 include the lungs, small intestine, gallbladder, kidneys, testes, thyroid, adipose tissue, heart muscle, vagina, breasts, ovaries, and pancreas [7]. The symptoms of COVID-19 include pneumonia, fever, fatigue, intestinal ischemia, and diarrhea[8, 9]. Many studies have reported gastrointestinal (GI) symptoms in COVID-19 patients, including diarrhea, nausea, vomiting, anorexia, abdominal pain, acid reflux, upper GI bleeding, haematochezia, constipation, and melena[10]. Besides, angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2, is highly expressed in intestinal tissue. Moreover, fecal samples from COVID-19 patients have tested positive for SARS-CoV-2 RNA testing. Thus, there is potential for fecal-oral transmission, with SARS-CoV-2 Likely impacting the GI tract[2].

As a part of the innate immune system, type III interferon (IFN-λ) mediates antiviral responses in the epithelial barrier. Moreover, IFN-λ receptor (IFN-λR)1 is preferentially expressed by the epithelial cells of the respiratory, intestinal, and reproductive tracts. The antiviral role of IFN-λ in intestinal infection has focused, long-lasting, and non-inflammatory characteristics. Consequently, many studies have explored the potential of applying IFN-λ in COVID-19[11]. Continuing research on SARS-CoV-2 showed that it is also expressed in the intestinal tract. In particular, IFN-λ might have crucial roles in SARS-CoV-2 intestinal infection.

In this review, we summarize the invasion and escape mechanisms of SARS-CoV-2, and both the direct and indirect influence of SARS-CoV-2 on intestinal homeostasis. We also consider how IFN-λ impacts intestinal infections of SARS-CoV-2, and focus on the new functions of IFN-λ in COVID-19 with intestinal symptoms.

SARS-CoV-2

Coronaviruses belong to the subfamily coronavirinae of the coronaviridae family, and have caused three pandemic outbreaks globally[12]. The subfamily coronavirinae consists of four genera; α, β, γ, and δ-coronaviruses. α and β-coronaviruses usually cause respiratory illness in humans and gastroenteritis in animals. In comparison, γ and δ-coronaviruses mostly infect birds, and, sometimes, mammals. According to genome sequence analysis, SARS-CoV-2, which was the third virus to create a pandemic, is classified in the β-coronavirus genome[12].

The four structural proteins of SARS-CoV-2 are essential for infections to occur. They consist of the spike (S) surface glycoprotein, membrane (M) protein, envelope (E) protein, and nucleocapsid (N) protein. The S protein is cleaved by host proteases, and allows SARS-CoV-2 to attach to host cells[13]. Both E and M proteins induce S protein to reside in the Golgi/ER Golgi intermediate compartment (ERGIC) compartment, and subsequently, together, regulate N-glycosylation maturation of the S protein [14]. N structural proteins are responsible for viral gene binding, compression, and packaging. In addition, they contribute to RNA transcription, and achieve the innate immune escape of the host through the N protein directly targeting and sequestering G3BP1 and host mRNA[15]. Once infected, SARS-CoV-2 mainly induces T cell responses; however, the generation of high-titer neutralizing antibodies by B-cell expansion and maturation might be limited[16,17]. To explore potential treatment

directions of SARS-CoV-2, it is important to learn its route of infection and mechanisms of immune evasion.

Infection mechanism of SARS-CoV-2

Zhou *et al*[18] first confirmed that SARS-CoV-2 only infects HeLa cells that express ACE2, which is used as a SARS-CoV-2 receptor in patients[18]. Although there are two independent ways to mediate the infection of SARS-CoV[19,20], the entry of SARS-CoV-2 involves ACE2-receptor-mediated transmembrane serine protease 2 (TMPRSS2)-dependent membrane fusion[21] (Figure 1).

SARS-CoV-2 is an enveloped virus containing a large N encapsidated positive sense RNA genome. S trimers with closed and open prefusion structures usually protrude from the lipid bilayer of SARS-CoV-2. They are distributed randomly on the viral surface to bind to the receptor, ACE2, and, subsequently, mediate viral uptake and fusion. The receptor binding domain (RBD) site is occluded by three copies of the N-terminal domain when in the closed prefusion. In the open prefusion, one or multiple RBDs lift up to expose the receptor binding site[22]. There are two main subunits, S1 and S2. S1 binds ACE2, while S2 triggers membrane fusion of the virus to the cell. In a typical SARS-CoV-2 infection process, S trimers must be activated by cellular protease-mediated cleavage at two distinct sites, S1/S2 and S2'. S2' is responsible for triggering virus-cell membrane fusion. TMPRSS2 is expressed on the cell surface, and activates S trimers, which promote the virus to enter the plasma membrane in a pH-independent way [23]. Finally, the encapsidated genome buds into the ERGIC to form virions, which are trafficked to the plasma membrane and released[22] (Figure 1).

Evasion mechanism of SARS-CoV-2

The first immune evasion of SARS-CoV-2 is related to the delayed occurrence of IFN-I[24]. As the first line of host defense against virus infections, IFN-I response is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) *via* host pattern recognition receptors (PRRs). SARS-CoV-2 RNA is detected by various cytosolic sensors, including retinotic acid-inducible gene 1 (RIG-I/DExD/H-box helicase 58) and melanoma differentiation-associated gene 5 (IFN induced with helicase C domain 1). These sensors induce an antiviral signaling cascade to occur[25]. A subset of IFN-stimulated genes (ISG) is produced by the IFN-I response. These ISGs, along with other downstream molecules controlled by IFN-I, directly inhibit virus replication and recruitment, and activate diverse immune cells[26]. However, viral proteins (NSP3/papain-like protease) encoded by the SARS-CoV-2 genome selectively cleave interferon regulatory factors (IRF) 3 protein directly during SARS-CoV-2 infection, resulting in a blunted Type-I IFN response[27]. M proteins on SARS-CoV-2 also target mitochondrial antiviral signaling proteins (MAVS), and impair the aggregation and recruitment of downstream signaling components[28]. In parallel, stress granule analogues, formed by the N protein, competitively bind G3BP1 to inhibit the innate immune response[15]. Furthermore, SARS-CoV-2 nsp13 (helicase), nsp14 (exonuclease), nsp15 (endoribonuclease), and accessory protein orf6 block IFN-I production by antagonizing upstream signaling pathways[29,30].

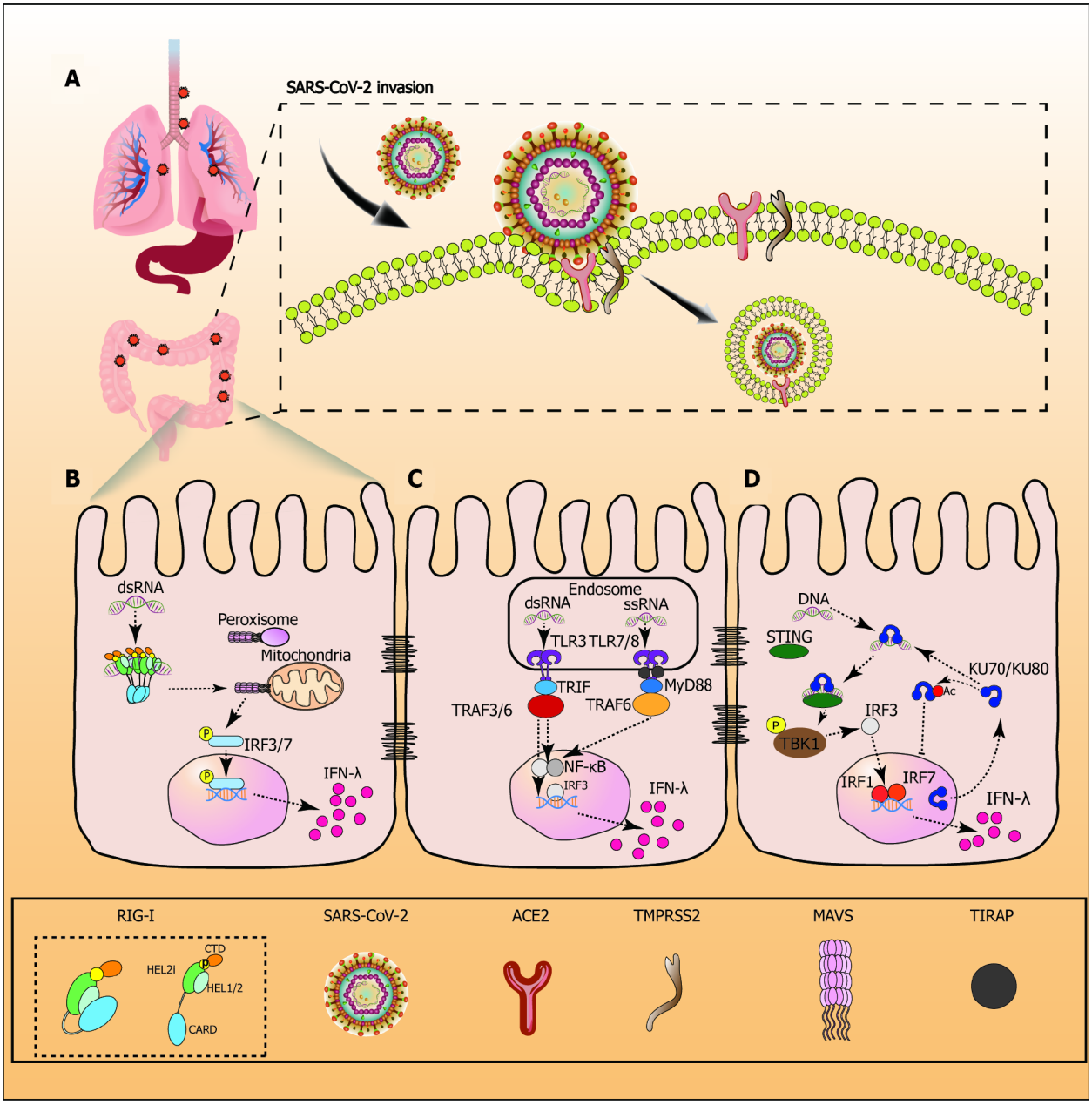
The second method of evasion is the upregulation of human inhibitory receptors, which modulate natural killer (NK) cell-mediated cytotoxicity. This process causes the exhaustion of NK cells, reducing their ability to clear viral infection[31].

Factors contributed to the severity of SARS-CoV-2

A recent meta-analysis of COVID-19 patients showed that physical activity could reduce the risk of COVID-19 infection. Physical activity strengthens the immune system, and allows cardiopulmonary and musculoskeletal adaptations[32]. Moreover, adherence to a high-quality healthy diet has been linked to a reduced risk of COVID-19 infection and lower hospitalization rates. Mediterranean-style dietary patterns are associated with a lower risk of death from respiratory infections, including COVID-19, while plant-based diets have been associated with lower COVID-19 infection rates[33]. Age is also an important factor in severe COVID-19 illness and its adverse consequences. Statistically, the case fatality ratio for COVID-19 increases with age[34]. There has also been widespread concern about whether pregnant women are more susceptible to SARS-CoV-2. However, to date, there are no data supporting an increased susceptibility to SARS-CoV-2 due to pregnancy. Nevertheless, the risk of death in pregnant women with COVID-19 is significantly increased[35]. There is also a nonlinear association between ambient temperature and the risk of infection with SARS-CoV-2. Specifically, the risk of infection with SARS-CoV-2 increases significantly at temperatures between 0 and 10 °C[36].

INTESTINAL INFECTION OF SARS-CoV-2

As a receptor of SARS-CoV-2, ACE2 is expressed in most organs. Consequently, COVID-19 is capable of infecting multiple organs, in addition to the respiratory tract[37]. It has been confirmed that ACE2 is abundantly expressed in GI glandular epithelial cells[38]. There is also clinical evidence that the intestine is another target organ. When SARS-CoV-2 infects the GI tract, it disrupts gut microbiota and induces other GI symptoms. Current research on SARS-CoV-2 invasion of the gut is focused on (1)



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Figure 1 Infection mechanism of severe acute respiratory syndrome coronavirus-2 and retinotic acid-inducible gene 1. A: Process of severe acute respiratory syndrome coronavirus-2 invasion in the gastrointestinal tract; B: Retinotic acid-inducible gene 1 (RIG-I) contains a pair of caspase activation and recruitment domains (CARDs), an ATPase motor domain (Helicase domain) that consists of two RecA domains (Hel1 and Hel2), and an alpha-helical insertion domain (Hel2i) connected *via* a Pincer motif (P) and a C-terminal domain. When RIG-I is in the inactivated state it clasps the CARDs against the surface of Hel2i. Once engaged with viral RNA, these pathogen RNAs trigger conformational changes that anchor the RNA tightly inside the RIG-I receptor and trigger the release of the CARDs[91]. Activated RIG-I interacts with mitochondrial antiviral signaling proteins to recruit phosphorylated interferon regulatory factor (IRF)3/7 in the nucleus, subsequently inducing interferon-λ (IFN-λ) production; C: Toll-like receptor (TLR) 3 interacts with dsRNA at opposite ends of the horseshoe ring in the endosome. TLR3 recruited TIR-domain-containing adapter-inducing interferon-β (TRIF) *via* the myeloid differentiation primary response 88 (MyD88) independent pathway. TRIF then recruits and activates the tumour necrosis factor receptor associated factor (TRAF) 3/6, inducing the recruitment of IRF3 and nuclear factor kB (NF-κB) in the nucleus. After TLR7/8 recognizes viral ssRNA in the endosome, the receptor-proximal membrane protein TIRAP detects the dimerized TIR domain of TLR7/8, and stimulates MyD88 to interact with it. MyD88 subsequently recruits TRAF6, leading to the recruitment of IRF3 and NF-κB in the nucleus. Once IRF3 binds to the promoter, IFN-λ gene expression is initiated[92,93]; D: Ku80 is co-localized with Ku70. Ku70 translocates from the nucleus to the cytoplasm, and recognizes cytosolic viral DNA. Subsequently, Ku70 with the DNA interacts with stimulator of interferon genes to phosphorylate TANK-binding kinase 1. Finally, IRF3 is activated, and produces the strong expression of IRF1and IRF7, which induce IFN-λ expression. Acetylation increases Ku70 accumulation in the cytoplasm and promotes DNA-mediated Ku70-dependent IFN-λ1 induction[94]. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IRF3/7: Interferon regulatory factor 3/7; IFN-λ: Interferon-λ; TRAF: Tumour necrosis factor receptor associated factor; TLR: Toll-like receptor; TRIF: TIR-domain-containing adapter-inducing interferon-β; NF-kB: Nuclear factor kB; TBK1: TANK-binding kinase 1; STING: Stimulator of interferon genes; MyD88: Myeloid differentiation primary response 88.

changes to the gut microbiome; and (2) whether GI symptoms are caused by direct infection of SARS-CoV-2 or the consequence of a systemic immune activation.

Impact of SARS-CoV-2 on the gut microbiome

The gut microbiome consists of bacteria, fungi, viruses, and other microorganisms that dynamically interact with environmental factors to shape the intestinal mucosal immune system[39]. The intestinal bacteria of healthy individuals are mainly composed of Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes. Intestinal microbiota is highly resilient to external disturbances, enabling the host to retain key species for a long time and maintain intestinal homeostasis. However, severe external influences cause microbial ecosystems to move from a stable state to an unhealthy stable state associated with disease[40]. A comparison of gut microbiota in COVID-19 patients and healthy people showed that the relative abundance of gut bacterial groups was higher in COVID-19 patients (including *Ruminococcus gnavus*, *Clostridium ramosum*, *Coprobacillus*, *Akkermansia muciniphila*, and *Eggerthella lenta*). Furthermore, the abundance of *Alistipes shahii* was comparatively lower in COVID-19 patients, along with several butyrate producers (including *Roseburia intestinalis*, *Eubacterium hallii*, *Ruminococcus bromii*, and *Faecalibacterium prausnitzii*)[41]. Gut microorganisms regulate mucosal sites in the distal part of the intestine *via* metabolites. These metabolites access other organs through the bloodstream, to induce immunomodulatory, immunoglobulin, and anti-inflammatory effects[42]. One cohort study showed that the lower abundance of *B. adolescentis*, *F. prausnitzii*, *E. rectale*, *R. (Blautia) obeum* and *D. formicigenerans* in the COVID-19 cohort might be associated with the decreased secretion of anti-inflammatory cytokines or increased secretion of inflammatory cytokines[43]. Another clinical study found that GI infection with SARS-CoV-2 differed to that without infection. Significant dysbiosis of the microbiome was documented in COVID-19 patients, with high levels of pathogenic bacteria[44]. In general, the intestinal microbiota of active GI infections with SARS-CoV-2 were mainly characterized by the enrichment of opportunistic pathogens and reduction of beneficial bacteria[45]. One study of COVID-19 patients showed that changes to the gut microbiome are correlated with the severity of COVID-19. In particular, the abundance of *Coprobacillus*, *Clostridium ramosum*, *and Clostridium hathewayi* is correlated with the severity of COVID-19. Furthermore, *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacterodies massiliensis*, and *Bacteroides ovatus* downregulate the expression of ACE2 in the murine gut, correlating with SARS-CoV-2 load in fecal samples[46].

In addition to SARS-CoV-2 directly infecting the GI tract, it affects the functioning of the GI tract through other organs. For instance, Dhar *et al*[47] showed that gut microbiota affected pulmonary health *via* vital cross-talk between the gut microbiome and lungs which is called the "gut-lung axis." However, the gut-lung axis is bidirectional; consequently, when SARS-CoV-2 infects the lungs, the gut microbiome might also be affected, including disruption[47].

Systemic immune activation

When infected by SARS-CoV-2, activated CD8+ IEL migrate to the intestinal mucosa[48]. The infected cells block IFN signaling, and exhibit strong pro-inflammatory responses by strongly activating the nuclear factor kB (NF-κB)/tumour necrosis factor pathway. In parallel, bystander cells not infected by SARS-CoV-2 mount an IFN-mediated response[49]. Although human plasmacytoid predendritic cells (pDCs) are not infected by SARS-CoV-2, pDCs may be activated by SARS-CoV-2 through IRAK4 and UNC93B1-dependent pathways, inducing high levels of type I and type III IFNS[50].

In healthy cells, CCL5, CXCL1, CXCL10, and CXCL11 recruit immune cells and pro-inflammatory chemokines. However, these four chemokines were significantly upregulated in SARS-CoV-2-infected epithelial cells in a trial of biomimetic human gut-on-chip system[51]. Interestingly, in a clinical cohort study, a subset of COVID-19 patients with GI symptoms had small intestinal epithelial cells infected with SARS-CoV-2. Compared to patients without GI symptoms, key inflammatory genes [including IFN-γ, CXCL8, CXCL2, and interleukin (IL)-1B] were downregulated in these cells, and the frequency of pro-inflammatory dendritic cells was reduced in immune subsets[52]. The authors also recorded lower mortality among COVID-19 patients with GI symptoms. Thus, a stronger immune response was induced in SARS-CoV-2 infected human intestinal tissue, reducing the efficiency of viral replication[53]. A recent study of rhesus monkeys infected with SARS-CoV-2 proposed that antiviral immunity was activated and the inflammatory response was enhanced at the early stage of intestinal infection. This response damages the intestinal barrier; however, the intestine was repaired and inflammatory response decreased at the later stage, leading to mild intestinal pathological damage[54]. Intestinal IFN-related antiviral response and neutrophil related pathways are activated at the early stage. This process damages Paneth cells and the intestinal barrier, enhancing the inflammatory response. Nevertheless, the increasing number of Paneth cells and B cells that inhibit neutrophil-chemotactic cytokines CXCL8 and IL-1B at the later stage, repair the homeostasis of the intestinal barrier, inhibiting inflammation and tissue damage[54].

TYPE III IFN

IFNs interfere with viral replication and induce inflammation, and are essential for mobilizing immune responses to SARS-CoV-2 infection[55]. There are three types of IFNs, and each has its own distinctive receptors. Type I IFN consists of at least 32 functional subtypes, including IFN-α subtypes (13 in humans and 14 in mice), IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ξ[56]. The type I IFN receptor (IFNR) is mainly expressed in immune cells. IFN-α/β responses to viral infections result in immunopathology. However, type I IFN also induces the infiltration of inflammation[57]. The mechanism of SARS-CoV-2 innate immune evasion depends on dysregulated IFN-I production, which contributes to robust early SARS-CoV-2 replication[31]. Type II IFN (IFN-γ) is a proinflammatory cytokine[58-60]. IFN-λ consists of four subtypes in humans (λ1, λ2, λ3, λ4), and its receptor is localized to epithelial cells and a subset of immune cells, including neutrophils. IFN-λ is widely believed to control pathologic microbes in the intestinal epithelium, and promotes the healing of the colonic epithelium without inducing an excessively strong inflammatory response[61].

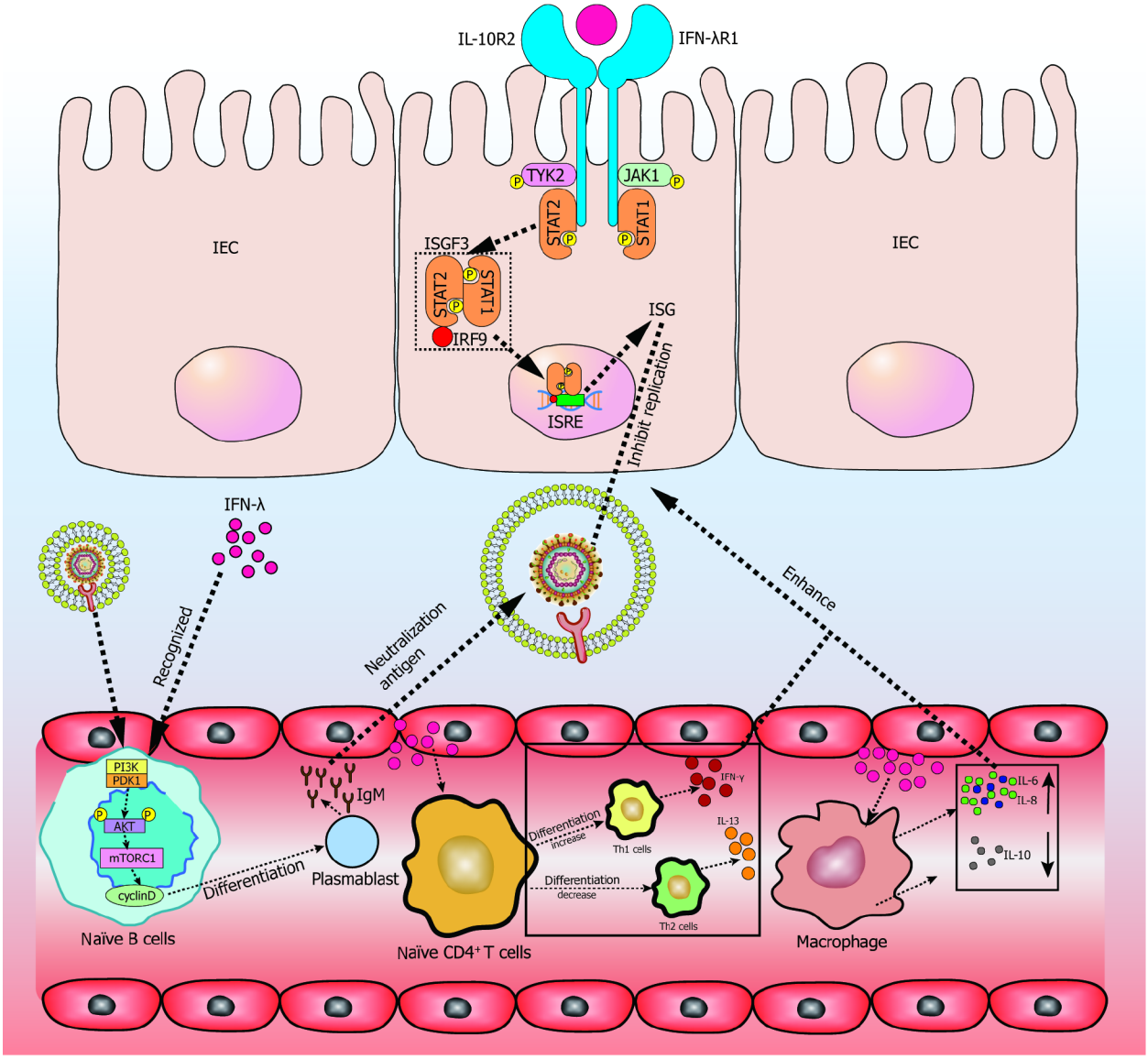
Immune responses triggered by PAMPs are key factors for pathogen defense. Differences in the location of PAMP recognized by some PRRs affect the type of IFN produced. PRRs in cytosol include RIG-I like receptors (RLRs), cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), and Ku70, whereas PPRs in the endosome are toll-like receptors (TLRs)[62]. cGAS mainly induces the expression of pro-inflammatory cytokines and the IFN-I gene through the stimulator of interferon genes (STING)-TANK-binding kinase 1 (TBK1)-IRF3/NF-κB signaling pathway. In comparison, RLRs, TLRs, and Ku70 induce the IFN-λ gene[63]. The downstream signaling pathways of IFN-λ is similar to IFN-I, which generates an antiviral response through the JAK-signal transducer and activator of transcription (STAT) pathway. In mitochondria or peroxisome, RLRs interacts with the MAVS, resulting IRF 3 and 7 being recruited into the nucleus. IRF3/7 binds to the promotor of IFN-λ to initiate the gene expression of the IFN-λ family. Activated TLR7, 8 and 3 signal is transduced by Myeloid differentiation primary response 88 and TIR-domain-containing adapter-inducing interferon-β. This process results in the recruitment of IRFs and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) in the nucleus. Subsequently, the expression of IFN-λ gene is enhanced[64]. The cytoplasmic translocation of Ku70 is the first step in initiating IFN-λ1 secretion, which facilitates the interaction between STING and Ku70. Activated STING promotes TBK1 phosphorylation, which activates IRF3. Since IRF3 is activated first, it produces the strong expression of IRF1 and IRF7, which are not endogenously expressed in cells. Subsequently IFN-λ1 is induced in the nucleus[65] (Figure 1).

In contrast to the type I IFNR, the IFN-λR is preferentially expressed by epithelial cells and neutrophils. The IFN-λR consists of IFN-λR1 (also called IL-28Rα) and IL-10R2. IFN-λR1 specifically binds IFN-λ, whereas IL-10R2 binds with cytokines in the IL-10 family. When IFN-λ signaling is activated by IFN-λ, the receptor-associated kinases JAK1 and TYK2 phosphorylate STAT1 and STAT2. IFN-stimulated gene factor 3 (ISGF3) is activated by IFN-λ, and is a trimeric complex that is formed by phosphorylated STAT1, STAT2, and IRF9. ISGF3 binds IFN-stimulated response elements in the nucleus to promote the transcription of hundreds of antiviral effector ISGs[66]. In addition to the STAT1-dependent pathway, IFN-λ promotes tissue healing by the upregulation of collagens *via* the MAPK-dependent pathway[67] (Figure 2).

IMPACT OF THE TYPE III IFN ON SARS-CoV-2 INFECTION OF THE GUT

Diarrhea is the most common GI symptom in COVID-19 patients. It is associated with changes to gut microbiota, malabsorption, and inflammation. It is also associated with the release of virulent proteins and toxins, and viral-induced intestinal fluid and electrolyte secretion. Of note, calprotectin, an inflammatory hallmark, was secreted by infiltrated neutrophils in COVID-19 patients with diarrhea[10].

The rapid initiation of the innate immune response after pathogen encounter is the result of infection and survival of the host. Highly expressed IFNs induce the secretion of ISGs, which is a hallmark of the innate immune response. ISGs induce various cell-intrinsic antiviral responses, such as blocking the translation and induction of apoptosis, while recruiting of immune cells and stimulating effector functions[68]. Compared to IFN-I, the effects of IFN-λ are delayed but last longer. IFN-λ is transcribed and translated at higher rates in intestinal organoids. Furthermore, IFN-λ is preferentially induced on the mucosal surfaces of both the intestine and lung[69]. Furthermore, cells cultivated under polarizing conditions exhibit high IFN-λ responsiveness[70]. Pretreatment with IFN-λ in Vero E6 cells showed dose-dependent inhibition of SARS-CoV-2[71]. IFN-λ promotes epithelial healing in patients infected with SARS-CoV-2 with acute colonic inflammation and tissue damage[72]. Moreover, IFN-λ treatment directly improves the proliferation and regeneration ability of differentiated GI epithelial cells through Lgr5+ intestinal stem cells (ISC), leading to the recovery of intestinal barrier integrity[73]. Furthermore, IFN-λ protects epithelial cells from enteric viral infections, indicating that IFN-λ could be used to treat SARS-CoV-2 infection of the GI tract.



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Figure 2 When interferon-λ is recognized in intestinal epithelial cells, JAK1 and TYK2 phosphorylate and activate phosphorylated signal transducer and activator of transcription 1 and signal transducer and activator of transcription 2. Interferon (IFN)-stimulated gene factor 3 is formed by phosphorylated signal transducer and activator of transcription 1, signal transducer and activator of transcription 2, and interferon regulatory factor 9. It then binds to IFN-stimulated response elements in the nucleus to promote the transcription of IFN-stimulated genes (ISGs). ISGs inhibit the RNA replication of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Recognition of IFN-λ in naïve T cells promotes the differentiation of Th1 cells while inhibiting Th2 cells, resulting in increased IFN-γ and lower interleukin (IL)-13. In peripheral mononuclear blood cells (PMBCs), IFN-λ induces higher IL-6 and IL-8, while reducing IL-10. The effects of IFN-λ on naïve T cells and PMBCs enhance the antiviral effect of ISGs in intestinal epithelial cells. IFN-λ and pathogens are co-recognized by naïve B cells, and promote the differentiation of naïve B cells to plasmablasts through the phosphoinositide 3-kinase-phosphoinositide dependent kinase-1 pathway. This action produces large amounts of immunoglobulin M (IgM). IgM acts as a neutralizing antibody in the intestinal tract of SARS-CoV-2 infection. STAT1: Signal transducer and activator of transcription 1; STAT2: Signal transducer and activator of transcription 2; IL: Interleukin; IFN-λ: Interferon-λ; IEC: Intestinal epithelial cell; ISGF3: IFN-stimulated gene factor 3; IRF9: Interferon regulatory factor 9; ISRE: IFN-stimulated response element; ISG: IFN-stimulated genes; PI3K: Phosphoinositide 3-kinase; PDK1: Phosphoinositide dependent kinase-1; mTORC1: Mechanistic target of rapamycin complex 1; IgM: Immunoglobulin M.

IFN-λ-mediated anti-SARS-CoV-2 response

IFN has been confirmed to be the first line of innate immunity, producing an antiviral response when the virus infects the intestinal tract. SARS-CoV-2 infects, replicates, and implements de novo infectious virus production in human intestinal epithelial cells (IECs). Furthermore, IFN-λ has huge potential for treating SARS-CoV-2. One study showed that the JAK-STAT1 dependent pathway of IFN-λ is more critical for anti-SARS-CoV-2 activity than IFN-I. Pretreatment IECs with IFN-λ resulted in a substantial decrease in the number of SARS-CoV-2 infected cells and viral replication[74]. Type I and II IFN upregulate antiviral ISGs when the virus infects cells, with ACE2 mRNA expression simultaneously increasing. Nevertheless, IFN-λ upregulates antiviral ISGs, whereas ACE2 mRNA is only marginally elevated[75]. Furthermore, one of the mechanisms by which IFNs restrict viral replication is to inhibit the translation process, which inhibits the expression of structural proteins, with SARS-CoV-2 being

more sensitive to IFN-λ. Compared to type I IFN, IFN-λ maintained a more consistent anti-SARS-CoV-2 infection state, with viral replication being inhibited for more than 72 h after drug withdrawal[76]. As for exogenous IFN-λ, pretreated IFN-λ significantly reduces the SARS-CoV-2 burden in the air-liquid interface of the lung model. The therapeutic administration of IFN-λ is also effective at restricting SARS-CoV-2 production in cultured human lung cells[77,78]. Another vitro experiment showed that recombinant bovine IFN-λ produced in HEK-293 cells effectively prevents SARS-CoV-2 infection toward VERO cells[79].

Effect of IFN-λ mediated recruitment and activation on immune cells

It remains unclear whether IFN-λ directly or indirectly regulates the immune response after SARS-CoV-2 infection. To date, IFN-λ1-4 is considered to regulate the immune response, including the upregulation of IL-6, IL-8, and the downregulation of IL-10 in peripheral blood mononuclear cells (PBMCs)[79]. It also alters Th1/Th2 T-cell balance, reduces IL-13 production by T cells[80], and induces ISGs in B cells and plasmacytoid dendritic cells, as well as in CD8 T cells, especially effector memory cells (TEMRA)[81] (Figure 2). As an anti-inflammatory factor, IL-10 induction in macrophages provides a powerful pathogen immune escape mechanism, which causes chronic infection[82]. IFN-λ stimulates a stronger innate immune response by upregulating IL-6 and IL-8, and by reducing IL-10 secretion in PBMCs, which promote an antiviral response[79]. *In vivo*, IFN-λ promotes the differentiation of initial T cells to Th1 cells, rather than Th2 cells, thus activating a stronger immune response, which combats the invasion of the gut by pathogens[80]. Furthermore, in the intestinal tract (unlike IFN-I), IFN-λ exhibits a compartmentalized response to virus infections because of the restricted expression of IFN-λR, which is preferentially expressed on IECs. IFN-λR expression has also been reported in NK cells, T cells, B cells, and pDCs[83]. Interestingly, IFN-λ induces the mechanistic target of rapamycin complex 1 pathway, leading to the differentiation of naïve B cells in plasmablasts by activating phosphoinositide 3-kinase *via* JAKs in an IRS-dependent, but STAT-independent, manner[84]. Thus, IFN-λ likely promotes the release of antibodies and increases the capability of antiviruses. These immune cells activate strong immune responses in the body to resist pathogen invasion and maintain intestinal homeostasis under IFN-λ regulation (Figure 2).

IFN-λ mediated regulation of intestinal inflammation

As a hallmark of intestinal inflammation in COVID-19 patients who have diarrhea, neutrophils are rapidly recruited from the blood after IEC infection. Neutrophils also restrict pathogen invasion through phagocytosis, reactive oxygen species (ROS), and the degranulation and release of cytotoxic antimicrobial molecules[85]. In contrast, excessive recruitment of neutrophils promotes inflammation. Thus, it is important to control the recruitment of neutrophils. Published studies show that IFN-λ provides a better balance of antiviral protection, with minimal inflammation and tissue damage. Specifically, IFN-λ in neutrophils inhibits the activation and phosphorylation of kinase AKT, which prevents the assembly of nicotinamide adenine dinucleotide phosphate oxidase complex NOX2 and decrease ROS in a JAK2-dependent way[86]. The effects of IFN-λ in neutrophils are independent of protein synthesis and transcription, only depending on the activation of AKT being inhibited[87]. Another study showed that human neutrophils optimally respond to IFN-λ in vivo, potentially inducing ISG levels to rise during inflammatory conditions by up-regulating IFN-λR1[88]. Mutual regulation between IFN-λ and neutrophils promotes intestinal resistance to SARS-CoV-2 invasion, and reduces the damage of intestinal tissue caused by inflammation.

CONCLUSION

IFN-λ potentially has a protective role in COVID-19 patients with intestinal infections. However, there is increasing evidence that IFN-λ has a complex dual role in the intestinal tract. Exposure to IFN-λ might increase intestinal permeability, which is associated with the disruption of junctional proteins[89]. Elevated IFN-λ is also related to lower numbers of Paneth cells and increased apoptosis of epithelial cells in the small intestinal[90]. In contrast, IFN-λ has been reported to exhibit an antiviral effect in the intestinal tract, and promotes the healing of IECs[71-73]. Although IFN-λ has an anti-SARS-CoV-2 effect in the intestinal tract, the mechanism remains unclear. Furthermore, how IFN-λ affects other intestinal cells (Paneth cells, goblet cells, and ISCs) and tight junctions in the intestine of SARS-CoV-2 patients requires investigation. Although IFN-λ has strong potential for use in COVID-19 intervention strategies, the mechanisms need to be elucidated first.

FOOTNOTES

Author contributions: Pan YY wrote the manuscript; Pan YY and Wang LC draw the pictures; Yang F collated the data; Yu M and Pan YY revised the manuscript.

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MINIREVIEWS

Comprehensive review on small common bile duct stones

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Abstract

Common bile duct stones are among the most common conditions encountered by endoscopists. Therefore, it is well researched; however, some items, such as indications for endoscopic papillary balloon dilatation (EPBD), safety of EPBD and endoscopic sphincterotomy in patients receiving dual antiplatelet therapy or direct oral anticoagulant, selection strategy for retrieval balloons and baskets, lack adequate evidence. Therefore, the guidelines have been updated with new research, while others remain unchanged due to weak evidence. In this review, we comprehensively summarize the standard methods in guidelines and new findings from recent studies on papillary dilation, stone retrieval devices, difficult-to-treat cases, troubleshooting during the procedure, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture.

Key Words: Choledocholithotomy; Choledocholithiasis; Common bile duct stones; Endoscopic papillary balloon dilation; Endoscopic sphincterotomy; Small common bile duct stones

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Core Tip: In this review, we comprehensively summarized the standard methods for patients with small common bile duct stones in guidelines and new findings from recent studies on papillary dilation, stone retrieval devices, difficult-to-treat cases, troubleshooting during the procedure, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture.

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INTRODUCTION

Cholangitis is the second or third most common cause of community-acquired bacteremia, with common bile duct (CBD) stones being the most common[1,2]. Recurrence of CBD stones is common, with 111 (11.3%) of 983 patients who underwent endoscopic sphincterotomy (EST) recurred during a median follow-up of 7.5 years, and the cumulative recurrence rates at 5, 10, 15, and 20 years were 8.5%, 12.5%, 19.1%, and 24.2%, respectively[3]. It is frequently encountered by endoscopists, and it is important to improve short-term outcomes and prevent the long-term recurrence of cholelithiasis. This review focuses on small CBD stones. Although the international definition of small CBD stones has not been established, we have followed the standard of approximately 10 mm in some studies[4,5]. We described papillary dilation, stone extraction, difficult cases, troubleshooting during stone extraction in small CBD stones, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture and summarized the European, American, and Japanese guidelines. Moreover, this review addressed the novel literatures on endoscopic papillary balloon dilatation (EPBD) dilation times to prevent post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP)[6], the duration of direct oral anticoagulants (DOAC) and dual antiplatelet therapy (DAPT) withdrawal to safely perform EST[7,8], EST with balloon dilation (ESBD), and the comparison of the effects of retrieval balloon and basket catheters for small CBD stone extraction[9,10].

COMPARISON OF EPBD AND EST

Papillary dilation is divided into EST and EPBD, and a nationwide administrative database of 61000 hospitalized patients with CBD stones throughout Japan reported that EST was performed in 89% of patients and EPBD in 11%[11]. Knowledge of the success rate of CBD stone removal and the incidence of short- and long-term complications is important when deciding between EST and EPBD.

Success rates of CBD stones clearance

A meta-analysis reported that EPBD has a lower incidence of total clearance of CBD stones and more frequent lithotripsy basket use than EST[12]. However, 11 of 14 references in this study included cases of CBD stones larger than 10 mm. Conversely, there were no significant differences in total clearance of CBD stones in another meta-analysis by Liu *et al*[13].

Yu *et al*[6] reported that both EST and EPBD have obvious effects in the treatment of bile duct stones with minor diameters (< 10 mm) and small numbers (< 3). The EPBD balloons used in that study were mostly 8 and 10 mm in diameter, especially those with 8 mm in diameter. Because a typical papillary dilation balloon is 8 mm in diameter, the indication for EPBD may be CBD stones up to 10 mm in diameter, considering the flexibility of the papillae. However, even for CBD stones > 10 mm, EPBD combined with endoscopic mechanical lithotripsy may have a success rate of stone retrieval comparable to that of EST[13]. Therefore, EPBD may be useful in cases of coagulopathy in which CBD stones are larger than 10 mm.

There is lack of evidence for the possibility of very small stone extraction without EST or EPBD. It has been reported that if ESWL results in stone fragment size of 3 mm or less, there is a likelihood that the stone will be spontaneously discharged without EST. Therefore, it is possible that stone extraction can be performed without EST or EPBD if the size is less than approximately 3 mm[14], however, there are no studies that have directly examined this issue. Therefore, in principle, EST and EPBD are recommended for stone extraction of CBD stones, as recommended by the European Society of Gastrointestinal Endoscopy (ESGE) and Japan Gastroenterological Endoscopy Society (JGES) guidelines; however, it is at the endoscopist's discretion whether to perform stone extraction without these procedures for very small stones[15,16].

Incidence of short-term complications

In cases of EPBD compared to EST, post-ERCP pancreatitis increased, bleeding decreased, and there was no significant difference in perforation or post-ERCP cholangitis. PEP and hemorrhage are likely to occur especially in approximately 10% and less than 0.1% of patients in the EPBD group, respectively; and in approximately 3% and 3% of patients in the EST group, respectively[12]. The total data in the meta-analysis has variation in the patient's background; however, it is consistent with that of a previous report[17].

PEP: PEP may be a short-term complication when selecting EST/EPBD. ESGE describes the following risk factors for PEP:

Patient-related definite risk factors include suspected sphincter of Oddi dysfunction, female sex, previous pancreatitis, and previous PEP. Procedure-related definite risk factors, such as difficult cannulation, pancreatic guidewire passage > 1, and pancreatic injection. Patient-related risk factors include younger age, non-dilated extrahepatic bile duct, normal serum bilirubin, absence of chronic pancreatitis, and end-stage renal disease. Procedure-related risk factors include precut sphincterotomy, pancreatic sphincterotomy, failure to clear bile duct stones, intraductal ultrasound, and biliary balloon sphincter dilation[18]. ESGE especially recommends prophylactic pancreatic stenting in selected patients at high risk for PEP (inadvertent guidewire insertion/opacification of the pancreatic duct and double-guidewire cannulation).

In a multicenter randomized control study, 117 patients with bile duct stones were treated with EPBD; after treatment, the incidence of pancreatitis among those patients reached 15.4%, and two patients died from post-treatment complications[19]. Incomplete dilation of the papilla, intramucosal bleeding, and local edema were considered the main causes of PEP due to EPBD. Conversely, several reports of randomized control trials or network meta-analyses suggested that there is no direct consequence between PEP risk and EPBD[20,21], and PEP usually occurs in the mild or moderate stage [12]. Recently, a network meta-analysis reported that 2 to 5 min of EPBD could decrease the incidence of PEP compared to short-term (< 2 min) EPBD. In addition, it was also reported to reduce PEP without increasing the occurrence of other early complications by extending the duration of balloon dilatation [6]. However, the underlying mechanism for this result remains unclear. A possible reason could be that the dilatation with a small diameter balloon or short duration could result in inadequate papilla expansion; thus, the common discharge channel for bile and pancreatic juice tended to be narrow after the operation[6]. That study did not examine EPBD longer than 5 min; however, another study found that 5-min EPBD increases PEP compared to EPBD of 0.5-3 min[22]. Although this is a study of EPBD combined with small-incision EST, it may be advisable to avoid EPBD for more than 5 min[22]. Therefore, we use a 2-3 min EPBD.

In recent years, diclofenac or diclofenac and sublingual nitrates have been reported to be useful for the prevention of PEP[23,24]. ESGE also recommends routine rectal administration of 100 mg of diclofenac or indomethacin immediately before ERCP in all patients without contraindications to nonsteroidal anti-inflammatory drug administration[18]. These methods were not available in 2004 when EPBD was abandoned by many endoscopists, especially in America, and combining such methods may reduce the incidence of PEP due to EPBD. Furthermore, EPBD may be even safer in Asians, as some race-based studies have shown no increase in PEP in Asian populations[12].

Bleeding: ESGE guidelines suggest that patients should be considered at increased risk of post-EST bleeding if at least one of the following factors is present: anticoagulant intake, platelet count < 50000/mm^3, cirrhosis, dialysis of end-stage renal disease, intraprocedural bleeding, and low endoscopist experience[18].

ESGE, JGES, and the American Society of Gastrointestinal Endoscopy (ASGE) guidelines treat antiplatelet medications almost similarly for EST/EPBD. DAPT is permitted in EPBD without drug withdrawal, whereas EST requires DAPT withdrawal. Withdrawal regimens are similar across guidelines, with thienopyridine requiring 5-7 day withdrawal and continuation of aspirin or cilostazol monotherapy[18,25,26]. However, each guideline treats anticoagulants in a slightly complex and different manner. Although it is necessary to evaluate the risk of embolism and procedural bleeding when antithrombotic agents are stopped in EPBD, warfarin can be continued if the PT-INR is within the therapeutic range. In EST, treatment with warfarin can be continued, whereas the PT-INR is within the therapeutic range in Japan and America. However, in Europe and America, it is recommended to discontinue warfarin 5 d before EST and replace it with heparin 2 d before EST, especially in patients at high risk of embolism in aortic or mitral valve replacement, atrial fibrillation, or any thromboembolic risk. Once hemostasis is confirmed, antithrombotic agents must be restarted postoperatively in America, the next day in Japan, and within 2 d in Europe. Warfarin should be resumed after the procedure, and heparin should be used in combination until the PT-INR returns to the therapeutic range[18,25,26]. However, it is difficult to summarize each country's guidelines accurately and concisely; therefore, please refer to each country's guidelines for details. In addition, in DAPT and DOAC, there is a paucity of evidence regarding the ability of guideline-guided withdrawal periods to prevent bleeding[7,8,25,27].

With regards to hemorrhage, Mirjalili and Stringer[28] identified 98 arteries near the major papilla and reported blood vessel distribution on endoscopy. According to their report, blood vessel distri-

bution in the 10 to 11 o'clock region was low at 10%-11%; thus, cutting in this region has a low risk of hemorrhage. The ESGE and Japanese EST guidelines have cited this article[16,18]. No trials have compared hemorrhage and perforation according to cutting direction; however, adding to the reports that bile ducts tend to run in the 11 to 12 o'clock direction in the papillary region, cutting in the 11 to 12 o'clock direction is considered safe, and thus recommended by Japanese EST guidelines[16].

Others: The superior sphincter extends to the bile duct on the lateral wall of the duodenum, and cutting beyond this area increases the risk of perforation. In relation to the papilla, it is believed that the superior margin of the papillary bulge coincides with the middle sphincter, which is considered the upper cutting limit (Figure 1). However, anatomical examinations may not necessarily be consistent with actual living bodies, and depending on the cutting direction, perforation can occur even if the superior margin of the papillary bulge is not reached; thus, due care should be exercised[16]. Moreover, there is no evidence comparing incision size to the incidence of procedural adverse events or therapeutic outcomes following EST[16].

The incidence of short-term cholecystitis after ERCP could be caused by resistance to initial antibiotics on admission[29], and the incidence of long-term cholecystitis and the recurrence of stones in CBD could be decreased by EPBD compared to EST[6,12]. EST causes significant damages to the Oddi sphincter, and post-EST sphincter dysfunction easily occurs[30]. Then, the reflux of intestinal contents such as digestive juices, food residue, and bacteria may increase the risk of biliary tract infection and stone recurrence[31,32].

To summarize the characteristics of EST and EPBD (Table 1), EST is superior in terms of PEP reduction and bile duct large stone retrieval, while EPBD is superior in terms of bleeding reduction, long-term cholecystitis, and bile duct stone recurrence. Based on these findings, we consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult.

ESBD: Ding *et al*[4] defined a tunnel from the distal bile duct to the papillary orifice as an extraction tunnel (SET). Based on the anatomical structure, the tunnel was divided into two segments, with the distal bile duct and the intradural portion of the sphincter of Oddi comprising the proximal segment, including the proximal ring, and the intraduodenal portion of the distal segment of the papillae, including the distal ring around the orifice. Conventional EST cuts the distal segment almost completely from the orifice to near the duodenal wall, EPBD extends the entire SET, and EST + EPBD (ESBD) shortens the SET by cutting the distal ring and extends the proximal ring. Therefore, this combination technique is suitable for accessing the wide opening of the SET from an anatomical perspective[4]. In this study, ESBD was reported to reduce the number of treatments for complete stone removal, procedure time, use of mechanical lithotripters, and bleeding rate, and the incidence of PEP was reported to be comparable to that of EST. It has been reported that a small incision did not increase the risk of bleeding compared with non-EST, which might be attributed to a lower chance of injury to the major vessel in the papillary roof[20]. ESBD limits EST to small incisions, which may be the reason for reduced bleeding after ERCP. In a network meta-analysis, ESBD tended to be superior to EST in terms of successful stone removal in the first endoscopic session, the need for mechanical lithotripsy, and the risk of bleeding or perforation. However, none of these variables showed statistical significance[20]. Thus, ESBD may be superior to EST in overall efficacy and short- and long-term complications, and ESBD may be recommended over EST in the future; however, there is insufficient evidence to recommend ESBD over EST. Therefore, to justify updating the current guidelines, researchers will require more evidence that ESBD is superior to EST in terms of overall efficacy[20] and that ESBD may reduce the long-term recurrence rate of bile duct stones[33]. At this time, it is up to each endoscopist to decide whether to perform ESBD or EST.

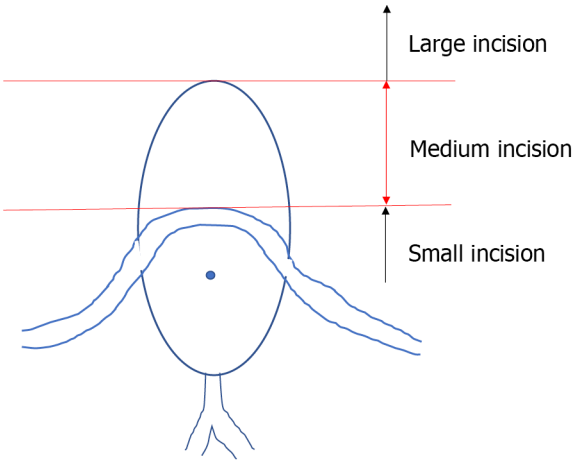
COMPARISON BETWEEN BALLOON AND BASKET CATHETER

A recent meta-analysis found that balloon catheters for cholelithiasis were superior to basket catheters for complete stone removal[9]. However, there are some limitations in the studies included in this meta-analysis. Three of the four studies included in the review were on small stones (≤ 10-11 mm), and three of these articles used a four-wire retrieval basket catheter. Four-wire retrieval basket catheters are less suited to retrieve small stones than an eight-wire retrieval basket catheters and retrieval balloon catheters. Therefore, we cannot conclude that the basket catheter is inferior to a balloon catheter in the case of small CBD stones[5,34-36]. One meta-analysis study only included these three studies, but its conclusions were similar to those of a previous meta-analysis[10]. Ozawa *et al*[5] reported that small stones (maximum diameter, 6 mm) are an independent risk factor for failed stone removal; in their study, the basket failed to grasp a small stone in eight cases, and in four of which, the stones were successfully removed after an exchange with a balloon catheter. Therefore, they suggested that a retrieval balloon catheter may be more appropriate than a basket catheter for removing small stones[5]. However, Ozawa *et al*[5] also used a four-wire basket.

Table 1 Short- and long-term complications of endoscopic papillary balloon dilatation/endoscopic sphincterotomy

	Short-term complications			Long-term complications	
	Bleeding	**PEP**	**Perforation**	**Cholecystitis**	**Recurrence of stones in CBD**
Incidence					
EST	3%	3%	Rare	EST > EPBD	EST > EPBD
EPBD	Less than 0.1%	10%	Very rare		
Prophylactic methods	Cessation of anticoagulant and antiplatelet agents referred to each country's guidelines; EST with cutting at approximately 11 o'clock direction	Diclofenac; Pancreatic stenting in selected patients at high risk for PEP; 2-3 min EPBD in patients with EPBD	There is no evidence comparing incision size, the incidence of procedural adverse events, and therapeutic outcomes following EST	We consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult	We consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult

CBD: Common bile duct; EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilatation; PEP: Post endoscopic retrograde cholangiopancreatography pancreatitis.



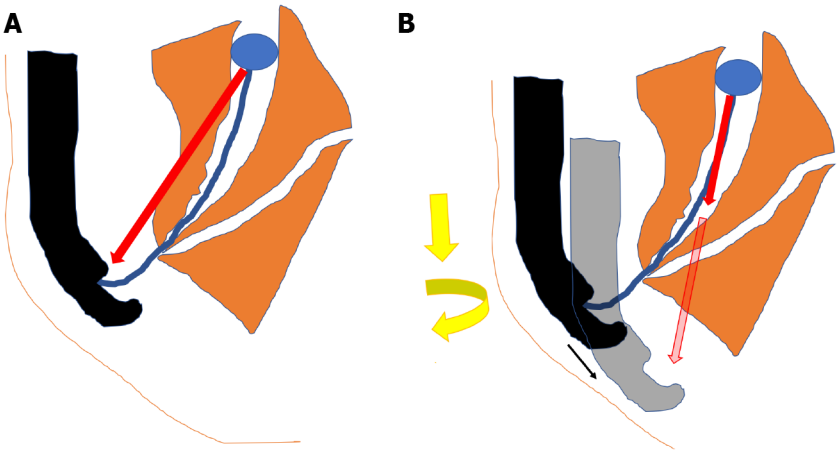
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Figure 1 The oral protrusion. Endoscopic sphincterotomy incision size. The risk of perforation increases when the incision exceeds the superior margin of oral protrusion.

Once a stone is captured in a basket, reliable extraction is usually ensured. More reliable traction associated with the basket catheter is cited as the main reason for its preferential use in Japan and Europe[5,9]. In the study by Ozawa *et al*[5], the balloon slipped past the stones and could not provide a sufficient traction force for stone extraction within 10 min in four patients in the balloon group, and the stones were successfully captured and withdrawn after exchange to the basket in all cases. However, a basket with a captured stone may occasionally become impacted at the papilla during extraction if the sphincterotomy is insufficient or if the stone is larger than estimated. According to the ESGE guidelines, the difference between balloon and basket catheters is slightly minimal, so endoscopists can use any of the two; meanwhile, according to the ASGE guidelines, the balloon catheter is highly recommended for safety issues related to basket impaction[18,37].

REMOVAL OF DIFFICULT SMALL BILE DUCT STONES

There are two main operations when retrieving CBD stones with a retrieval balloon or basket. First, the catheter was pulled with the right hand. The other is to apply right rotation and push on the endoscope and use the down angle with dial control, if necessary. The difference between the two is the direction of the force on the retrieval balloon or the basket. In the former, the retrieval balloon or basket faces the forceps hole at the endoscope tip, whereas, in the latter, they face the tip of the endoscope that is pushed in (Figure 2). The important basic rule is that the direction of the force applied to the catheter should coincide with the long axis of the bile duct, and one can choose the easier of the two methods to accomplish this.



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Figure 2 Basket/balloon catheter operations. A: Direction of force on the retrieval balloon or basket when pulling the catheter with the right hand; B: Direction of the force on the retrieval balloon or basket when applying right rotation and pushing the endoscope.

However, in cases with pockets in the lower part of the bile duct, stone extraction is difficult. Once a stone is impacted at the corner pocket, the balloon passes alongside the stone without removing it, and stone removal is often difficult, even after repeated attempts. Such cases can be handled by pushing the stone up to the middle of the bile duct and then grabbing it with a basket or by using a basket shaped to extract the stone out of the pocket, such as a disposable NT retrieval basket (VorticCatch V: Olympus Medical Systems, Japan) (Figure 3).

Furthermore, stones near the bifurcation of the gallbladder duct are difficult to grasp using a retrieval balloon or basket (Figure 4). Surgery is considered in these cases; however, they can be addressed with cholangioscopy, such as when in conjunction with electronic hydraulic lithotripsy (EHL)[15]. When it is difficult to grasp a CBD stone, a basket that directly grasps the stone under cholangioscopy is available [38].

Enteroscopy-assisted ERCP (eERCP) is often difficult in cases of surgically altered anatomy (SAA). In cases of SAA, endoscopic ultrasound-guided transmural drainage (EUS-TD) or percutaneous transhepatic biliary drainage (PTBD) may be effective alternatives (Figure 5)[39].

In a multicenter retrospective cohort study involving 98 patients (49 EUS-TD and 49 eERCP groups), technical success was achieved in 98% of patients in the EUS-TD group compared to 65.3% of patients in the eERCP group (OR 12.48, $P = 0.001$). EUS-TD had a significantly shorter procedural time (55 *vs* 95 min, $P < 0.001$). However, more complications of mild/moderate severity occurred in the EUS-TD group (20% *vs* 4%, $P = 0.01$). The length of stay was significantly longer in the EUS-TD group (6.6 *vs* 2.4 d, $P < 0.001$)[40]. PTBD is also a useful alternative, with a reported success rate of approximately 97%, but this method of stone removal may cause problems, such as drainage tube trouble or an increased number of sessions[41].

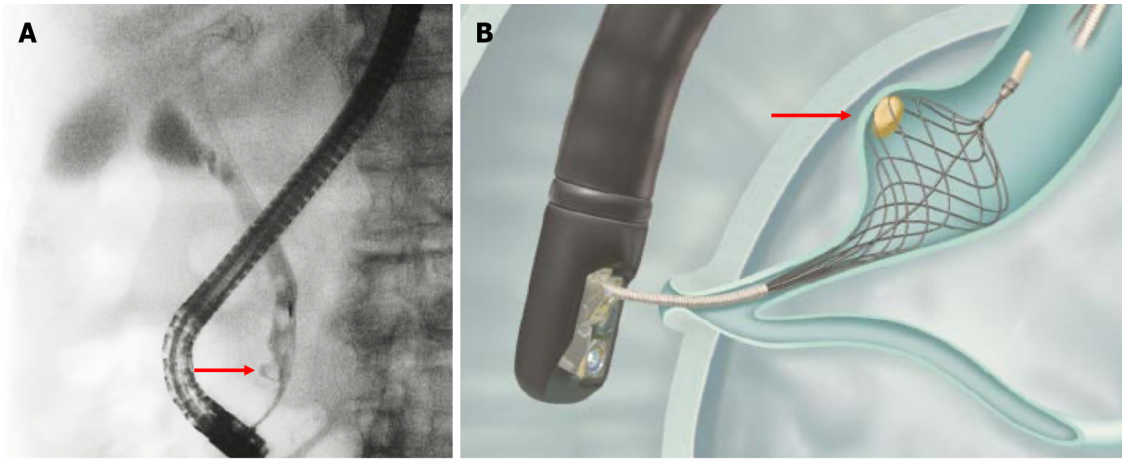
TROUBLESHOOTING DURING STONE REMOVAL

A serious drawback of basket catheters is that during stone extraction, the basket with the captured stone is impacted in the lower bile duct or papilla. When basket impaction occurs, the basket must first be opened and pushed upwards into the hepatic hilum. An attempt was made to curl the basket wires back and disengage the stone (Figure 6). If this technique fails, more complicated techniques, such as mechanical lithotripsy and intra-extracorporeal lithotripsy, are required[5]. To use a lithotripter, such as BML-110A-1 (Olympus Medical Systems, Tokyo) (Figure 7), which can be retrofitted to a basket catheter, the basket catheter is cut outside the body, the endoscope is removed from the body, and the wires of the basket catheter from the mouth are wrapped around the lithotripter. However, if the basket cannot be unmated even with a lithotripter, a cholangioscope can be helpful. The basket and grasped stone were visualized under the cholangioscope and crushed by an EHL or YAG laser (Figure 8).

SPECIFIC SITUATION

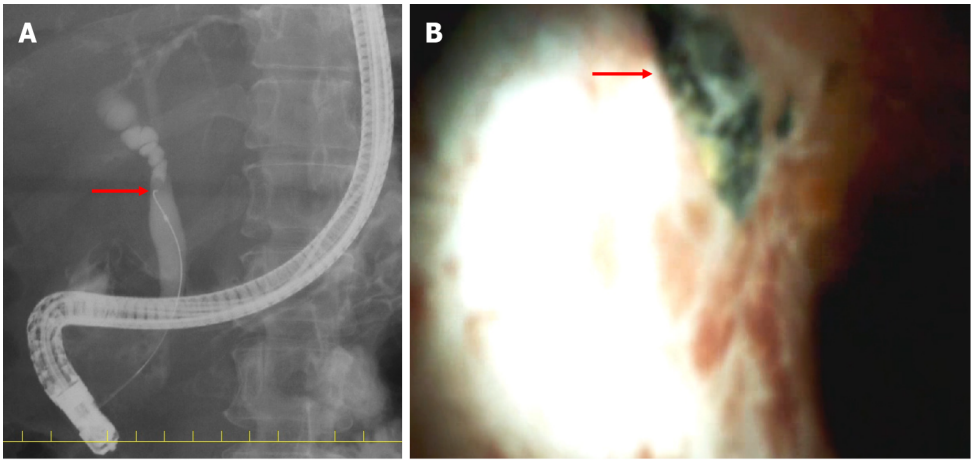
CBD stones complicated with cholangitis

The Tokyo Guidelines 2018 (TG18) and ASGE suggest that bile duct stone removal following EST in a single session may be considered in patients with mild or moderate acute cholangitis[42,43]. However,



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Figure 3 Stone in the lower common bile duct pocket. Red arrows indicate a stone in the lower common bile duct pocket. A: A case with stone in the lower common bile duct pocket; B: Disposable NT retrieval basket (VorticCatch V: Olympus Medical Systems, Japan).



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Figure 4 Stone stuck in the bifurcation of the gallbladder duct. Red arrows indicate a stone stuck in the bifurcation of the gallbladder duct. A: The stone got stuck in the bifurcation of the gallbladder duct, as seen by endoscopic retrograde cholangiopancreatography. The guidewire could not be inserted into the gallbladder duct because of the obstruction by a stone; B: A stone stuck in the bifurcation of the gallbladder duct as observed by cholangioscopy.

given that hemodynamically unstable or coagulopathy patients might not tolerate procedural bleeding or adverse events, decompression alone should be considered in this group[42,43]. PEP does not increase even in cases of complicated cholangitis[43]. TG18 suggested that endoscopic nasobiliary drainage (ENBD) or endoscopic biliary stenting (EBS) may be considered for biliary drainage according to the patient's background and preference. It should be borne in mind that if patients experience discomfort from transnasal tube placement, they are likely to remove the tube themselves, particularly in elderly patients. EBS is an internal drainage technique that does not cause discomfort or loss of electrolytes or fluids. In contrast, ENBD is an external drainage technique that allows monitoring or washing of bile *via* the transnasal tube, particularly if the bile is purulent[42]. The ESGE did not provide any recommendations for these[15]. We present a table summarizing each guideline, focusing on key points (Table 2).

CBD stones complicated with cholecystolithiasis

In the general population, CBD stones complicated with cholecystolithiasis commonly occurs. The established gold standard for the treatment of symptomatic cholecystolithiasis is laparoscopic cholecystectomy (LC), but the treatment option for CBD stones is yet to be clarified. CBD stones complicated with cholecystolithiasis can be treated with two-session minimally invasive and one-session feasible strategies. The former requires pre- or post-LC ERCP, whereas the latter requires LC plus intraoperative laparoscopic CBD exploration (LCBDE) or LC with intraoperative ERCP[44]. As per efficacy, morbidity, or mortality endoscopic and surgical techniques for extracting these stones are equally suitable[45]. However, one-session procedures usually result in a shorter hospital stay[15].

Table 2 Key points of each guideline

	JGES/TG18	ESGE	ASGE
Papillary dilatation	EST is standard	EST is standard	EST is standard
	EPBD is determined by age, scheduled RFA, antithrombotic medications, parapapillary diverticulum, reconstructed bowel, and stone diameter and number	In anticoagulant users and in cases of SAA, EPBD is an option for stones smaller than 8 mm	In anticoagulant users, in cases of SAA, and in cases of intradiverticular papilla, EPBD is an option
Cases of antithrombotic agents use (EST, high bleeding risk procedures)	ASA or CLZ alone may be continued	ASA or CLZ alone may be continued	ASA or CLZ alone may be continued
	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ
	Warfarin may be continued if in therapeutic range; DOAC is withdrawn on the EST day only	Warfarin stopped 5 d ago and LMWH was started 2 d ago (LMWH also stopped 24 h ago). DOAC stopped 48 h ago	Warfarin users can be treated urgently if INR < 2.5. DOACs should be discontinued prior to treatment, with a discontinuation period of twice the half-life. Heparin replacement is recommended in patients at high risk of thrombosis
	Resumed the next day	Warfarin or DOAC resume within 48 h. Warfarin is used with LMWH until the optimal concentration is reached	Resume at the end of the procedure if hemostasis is confirmed. However, evidence for DOACs and APAs are scant
Stone retrieval	No superiority of balloons and baskets is noted	Efficacy of balloons and baskets is almost equal	Recommend using balloons rather than baskets
Complicated cases of cholangitis	Patients who are hemodynamically unstable, coagulopathic, or receiving antithrombotic agents; it was believed that decompression alone should be considered.EBS and ENBD are almost equal	Not stated	Given that hemodynamically unstable patients might not tolerate procedural bleeding or adverse events, it was believed that decompression alone should be considered in this group as well as for patients who are coagulopathic and/or are receiving antithrombotic agents and those who would need to have anticoagulation resumed immediately after sphincterotomy (*e.g.*, patients with mechanical heart valves)

APA: Antiplatelet agent; ASA: Acetylsalicylic acid; ASGE: American Society for Gastrointestinal Endoscopy; CLZ: Cilostazol; DOAC: Direct oral anticoagulant; EBS: Endoscopic biliary stent; ENBD: Endoscopic nasobiliary drainage; EPBD: Endoscopic papillary balloon dilation; ESGE: European Society of Gastrointestinal Endoscopy; EST: Endoscopic sphincterotomy; JGES: Japan Gastroenterological Endoscopy Society; LMWH: Low-molecular-weight heparin; SAA: Surgically altered anatomy; TG18: Tokyo guideline 2018.

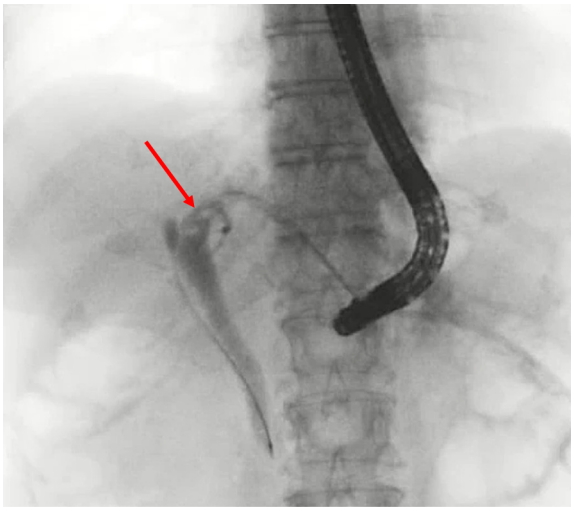
Moreover, a recent meta-analysis has demonstrated that the one-session procedure has a higher success rate than the two-session procedure[46].

For one-session procedures, many surgeons prefer the less invasive and less complicated transcystectomy approach, however, bile duct incision is recommended for dilated CBD, large diameter and multiple stones, impacted stones, and stones with intrahepatic localization[47,48]. It is recommended to start with transcystectomy and move unto exploration by bile duct incision if difficult[44,49]. Laparoscopic stone removal can be performed fluoroscopically or cholangioscopically. The use of a flexible cholangioscope is the most preferred method because of its accuracy and direct visual control. However, one-session procedure requires advanced laparoscopic techniques, a long learning curve, and specialized equipment, and these qualities may not exist in all treatment facilities[50-52]. ESGE recommends that transcystic or transductal exploration of the CBD is a safe and effective technique for removal of CBD stones in patients undergoing laparoscopic cholecystectomy, provided that local expertise and resources are adequate[15]. It is of note that results of surgical treatment of CBD stones, which are generally excellent in published reports, are usually from laparoscopic centers of excellence, however, there are hardly reports by less experienced surgeons. Therefore, the ESGE does not clearly state whether one-session or two-session procedure should be preferred.

There are no recent reports on laparoscopic surgery for small CBD stones, however, Huang *et al*[53], in their report on laparoscopic surgery for small CBD with CBD stones, indicated that it is safe and feasible for small CBD patients to perform LCBDE.

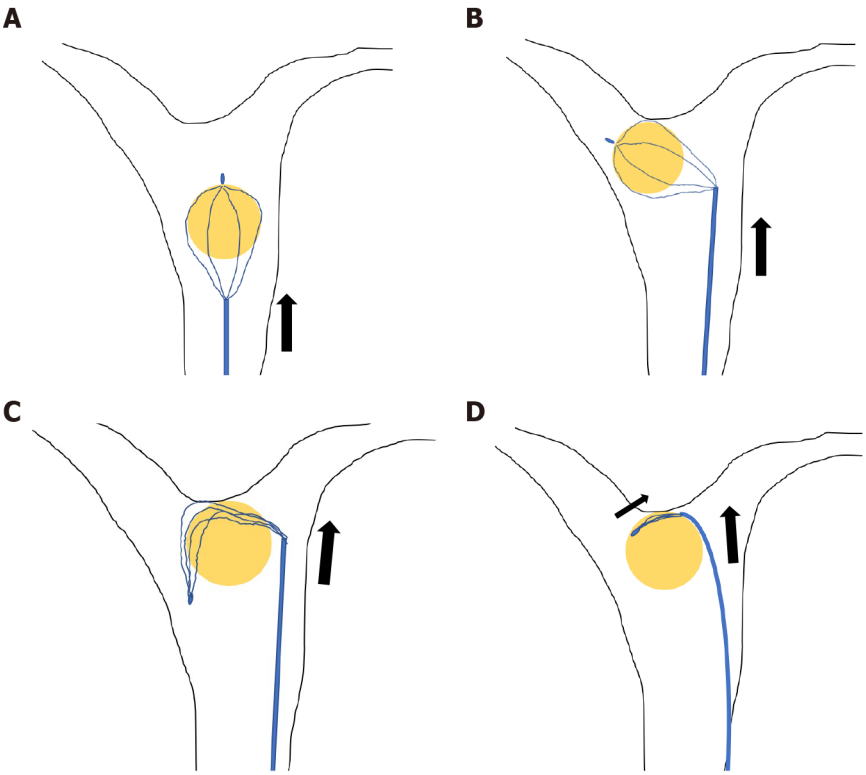
CBS stones complicated with distal biliary stricture

Few reports have been published on CBD stones extraction with distal biliary stricture[54,55], however, plastic stent(s)[56,57], covered self-expandable metallic stent(s) (cSEMS)[56-58], balloon dilation[59], and surgery[60] have been used for dilating bile duct stenosis. However, balloon dilation carries the risk of



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Figure 5 Case of total gastrectomy with RY reconstruction. Enteroscopy-assisted endoscopic retrograde cholangiopancreatography was unsuccessful; therefore, an endoscopic ultrasound-guided hepaticogastrostomy was performed. The red arrow indicates bile duct stones.



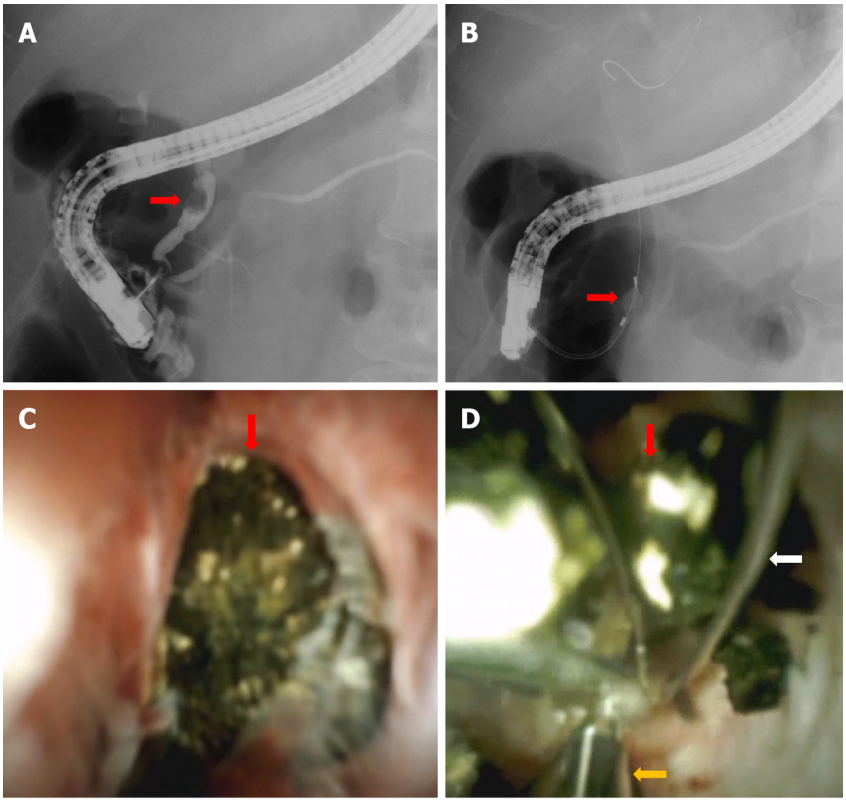
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Figure 6 Release of grasped stones. A: Push the basket catheter up into the hepatic hilum; B: Push further to invert the grasped stone; C: Push and deflect the basket wire; D: Close the basket while pushing the catheter.

bile duct injury. Therefore, when endoscopic stone extraction is performed for CBD stones with benign biliary stricture, it may be advisable to use multiple plastic stents or cSEMS for several months and perform endoscopic stone extraction after bile duct dilation is achieved[61]. Combining them with mechanical lithotripsy may also be useful[54]. Ogura *et al*[55] reported that transluminal stone extraction passing through the EUS-TD route, without passing through the distal bile duct might be useful. Reports of CBD stones with malignant biliary stricture are even more scarce, however, the safety of 6-8 mm balloon dilation for malignant biliary stricture has been reported[62]. In malignant biliary stricture with limited prognosis, stenting alone may be sufficient and stone extraction may not be necessary, however, balloon dilation for stone extraction may be considered in cases of short-term stent obstruction.



Figure 7 BML-110A-1 (Olympus Medical Systems, Tokyo). The authors have obtained the permission for figure using from the Olympus (Supplementary material).



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Figure 8 The case in which the basket could not be unmated even with the lithotripter. The basket and grasped stone were visualized under cholangioscopy and crushed using electronic hydraulic lithotripsy (EHL). The red arrows indicate common bile duct (CBD) stones, white arrow basket catheter, and orange arrow EHL probe. A: CBD stone; B: CBD stone grasped by basket; C: CBD stone grasped by basket as seen by cholangioscopy; D: CBD stone crushed with EHL.

CONCLUSION

While EST is the standard treatment for papillary dilatation, EPBD is also a viable option for younger patients who wish to reduce the risk of long-term recurrence and patients with coagulopathy. EPBD is considered to have a lower risk of bleeding and perforation than EST. Several methods have been recently proposed to reduce PEP, the greatest weakness of EPBD. We would also like to focus on ESBD, which should be the subject of future research.

For small stones in the CBD, it is not necessary to strictly distinguish between the retrieval balloon and the basket; however, if one device cannot remove the stone, it is recommended to use the other. In cases of pockets in the lower bile duct, Voltic catch V is also useful. It is also important to gain experience in the use of EUS-TD, lithotripter, and cholangioscopy to deal with troubleshooting such as stones stuck in the basket and difficult cases of stone retrieval.

In cases of complicated cholangitis, stone retrieval can be performed in mild or moderate cases in a single session. In severe cases, decompression alone should be considered, and EBS is generally

recommended. Cases of CBD stones complicated with cholecystolithiasis that are scheduled for one-session surgical treatment or CBD stones complicated with distal biliary stricture should be treated in facilities with adequate experience and equipment.

FOOTNOTES

Author contributions: Masuda S and Koizumi K were major contributors to writing the manuscript; Shionoya K, Jinushi R, Makazu M, Nishino T, and Kimura K designed the outline and coordinated the writing of the manuscript; Sumida C, Kubota J, Ichita C, Sasaki A, Kobayashi M, Kako M, and Haruki U provided input for writing the paper.

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MINIREVIEWS

Liver transplantation in the management of cholangiocarcinoma: Evolution and contemporary advances

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Abstract

Cholangiocarcinoma (CCA) is an aggressive malignancy arising from the biliary epithelium. It may occur at any location along the biliary tree with the perihilar area being the most common. Prognosis is poor with 5-year overall survival at less than 10%, typically due to unresectable disease at presentation. Radical surgical resection with clear margins offers a chance of cure in patients with resectable tumours, but is frequently not possible due to locally advanced disease. On the other hand, orthotopic liver transplantation (LT) allows for a radical and potentially curative resection for these patients, but has been historically controversial due to the limited supply of donor grafts and previously poor outcomes. In patients with perihilar CCA, within specific criteria and following the implementation of a protocol combining neoadjuvant chemoradiation and LT, excellent results have been achieved in the last decades, resulting in its increasing acceptance as an indication for LT and the standard of care in several centres with significant experience. However, in intrahepatic CCA, the role of LT remains controversial and owing to dismal previous results it is not an accepted indication. Nevertheless, more recent studies have demonstrated favourable results with LT in early intrahepatic CCA, indicating that, under defined criteria, its role may increase in the future. This review highlights the history and contemporary advances of LT in CCA, with particular focus on the improving outcomes of LT in intrahepatic and perihilar CCA and future perspectives.

Key Words: Cholangiocarcinoma; Klatskin tumor; Liver transplantation; Liver cancer; Liver resection; Neoadjuvant therapy

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Core Tip: Cholangiocarcinoma (CCA) is an aggressive malignancy with poor prognosis. Radical surgical resection with clear margins may offer a chance of cure but is frequently not possible due to locally advanced disease. In the last two decades, within specific criteria, a protocolised combination of neoadjuvant chemoradiation and orthotopic liver transplantation (LT) has produced excellent results in patients with perihilar CCA, while favourable results have been shown with LT in early intrahepatic CCA in recent years. We review the history and contemporary advances of LT in CCA and discuss future perspectives.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignancy of the biliary epithelium. It is rare, affecting less than 6 persons per 100000 population[1,2]. CCA carries a poor prognosis with 5-year overall survival (OS) rates for all stages and subtypes at less than 10%[3-5]. CCA is further subdivided depending on the site of origin of the tumour in the biliary tract, with intrahepatic, perihilar (further subdivided by the Bismuth-Corlette classification) and distal variants recognised (Figure 1). These subtypes vary not only in their anatomical site, but also their biology with distinct phenotypes[6].

Complete surgical resection is the only curative therapy in CCA but is precluded in most individuals due to advanced disease at presentation. Unresectable disease has a median survival of between 6 mo to 1 year[7]. The type of surgical resection offered varies depending on the anatomic subtype of the CCA. Intrahepatic and perihilar CCAs mandate liver resections with or without excision of the extrahepatic bile ducts depending on the location and radiological[8] extent of invasion of the tumour. The risk of postoperative liver failure is largely dictated by the quality and volume of the future liver remnant which may prohibit resection in many patients. Techniques such as portal vein embolization and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) have been developed to extend the limits of resectability in liver surgery by inducing hypertrophy in the contralateral side of the liver (if/when disease-free), hence increasing the future liver remnant[9]. However, ALPPS is largely considered contraindicated in perihilar CCA (phCCA) owing to high perioperative mortality (48% at 90 d)[10]. For distal bile duct tumours, excision of the extrahepatic bile duct is combined with a pancreatoduodenectomy to achieve an adequate oncological margin.

With all of the above procedures, arteriovenous invasion of the tumour was historically seen as an absolute contraindication to resection with poor outcomes. More recently, both portovenous, caval and arterial reconstruction of hepatic, superior mesenteric and even the coeliac arteries have been described, with some series showing favourable long-term outcomes. Venous reconstruction can be performed with similar perioperative morbidity to conventional resection, whilst long-term survival is equivalent in some series[11]. Arterial reconstruction is associated with higher perioperative morbidity and mortality and significantly lower long-term disease-free and OS compared to standard resection, however, outcomes remain significantly superior to palliative care[12,13].

CCA may also involve both liver lobes either as a single mass at the hilum (Bismuth-Corlette type IV phCCA), as multicentric disease or by metastasis making disease unresectable without complete hepatectomy.

Despite this, most patients remain unresectable by virtue of locally advanced or distant metastatic disease, with a dismal median survival of 11.7 mo with palliative chemotherapy[7].

Liver transplantation (LT) has emerged as a potential solution to expand resectability of locally advanced CCA, with promising early patient outcomes. There is also new evidence that LT may confer superior patient outcomes even in technically resectable tumours[14]. This review aims to summarise the literature on the evolving role of LT in CCA.

HISTORY OF LIVER TRANSPLANTATION FOR MALIGNANCY

Initial indications for orthotopic liver transplants were focused on patients with malignancy[15]. Indeed, the second successful LT was performed for intrahepatic CCA (iCCA), by transplantation pioneer Thomas Starzl at the University of Colorado in 1963[15,16]. The recipient patient died on postoperative day 7 from respiratory failure and gastrointestinal bleeding. The early experience with LT unfortunately demonstrated prohibitively high perioperative mortality due to pulmonary emboli and haemorrhage, because of the veno-venous bypass and excessive anticoagulation used at the time. Coagulopathic

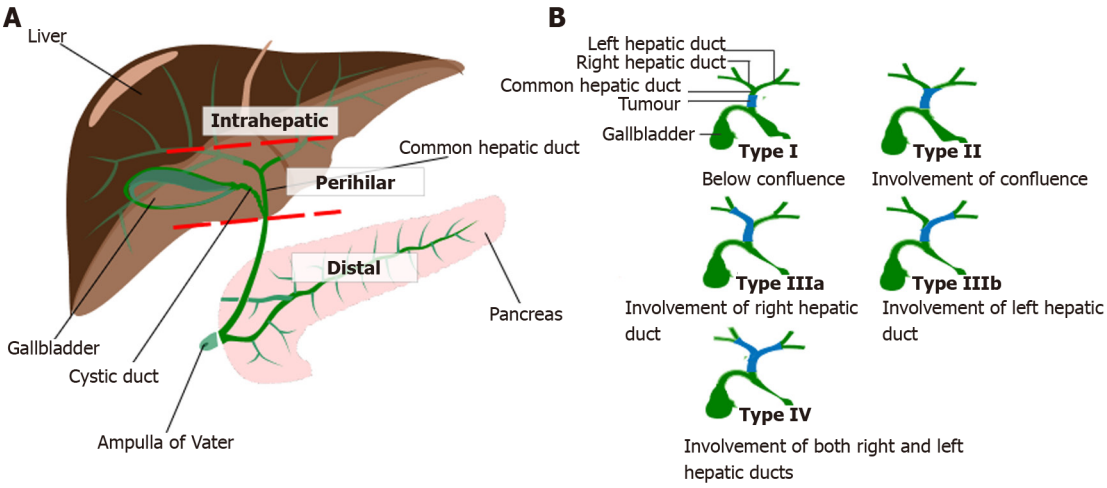


Figure 1 Classification of cholangiocarcinoma[62,71]. A: Anatomical classification of cholangiocarcinoma: Intrahepatic cholangiocarcinoma-proximal to second order bile ducts; Perihilar cholangiocarcinomas-between second order branches of right and/or left hepatic ducts and cystic duct confluence; Distal cholangiocarcinoma-between cystic duct confluence and Ampulla of Vater; B: Bismuth Corlette Classification of perihilar cholangiocarcinoma, https://creativecommons.org/licenses/by-sa/4.0/deed.en. Citation: Borakati A, Froghi F, Bhogal RH, Mavroeidis VK. Stereotactic radiotherapy for intrahepatic cholangiocarcinoma. *World J Gastrointest Oncol* 2022; 14: 1478-1489 [PMID: 36160742 DOI: 10.4251/wjgo.v14.i8.1478] and Wikimedia Commons. File: Bismuth corlette classification for perihilar cholangiocarcinomas.svg. 2020 Oct 9 [visited 3 February 2023]. Available from: https://commons.wikimedia.org/wiki/File:Bismuth_corlette_classification_for_perihilar_cholangiocarcinomas.svg.

complications were overcome with the routine use of thromboelastography to guide correction of clotting derangements in real-time. Veno-venous bypass was later used without anticoagulation and obviated by newer surgical techniques such as the 'piggy-back' method which retains the recipient inferior vena cava and does not necessitate complete clamping of this vessel and the resultant haemodynamic instabilities[17]. The other main driver of early postoperative mortality was biliary sepsis and obstruction. This was largely overcome by routinely performing biliary anastomoses over T-tubes or by using Roux-en-Y reconstructions.

The development of improved organ preservation solutions, most notably the University of Wisconsin solution in 1988[18], allowed greater cold ischaemic times with consequent reductions in vascular and biliary complications[19]. At present, liver malignancies indicated for LT in well selected patients include hepatocellular carcinoma (HCC), hepatic epitheloid hemangioendothelioma, hepatoblastoma and metastatic neuroendocrine tumours, while an increasing interest is developing about its role in CCA and metastatic colorectal cancer[20].

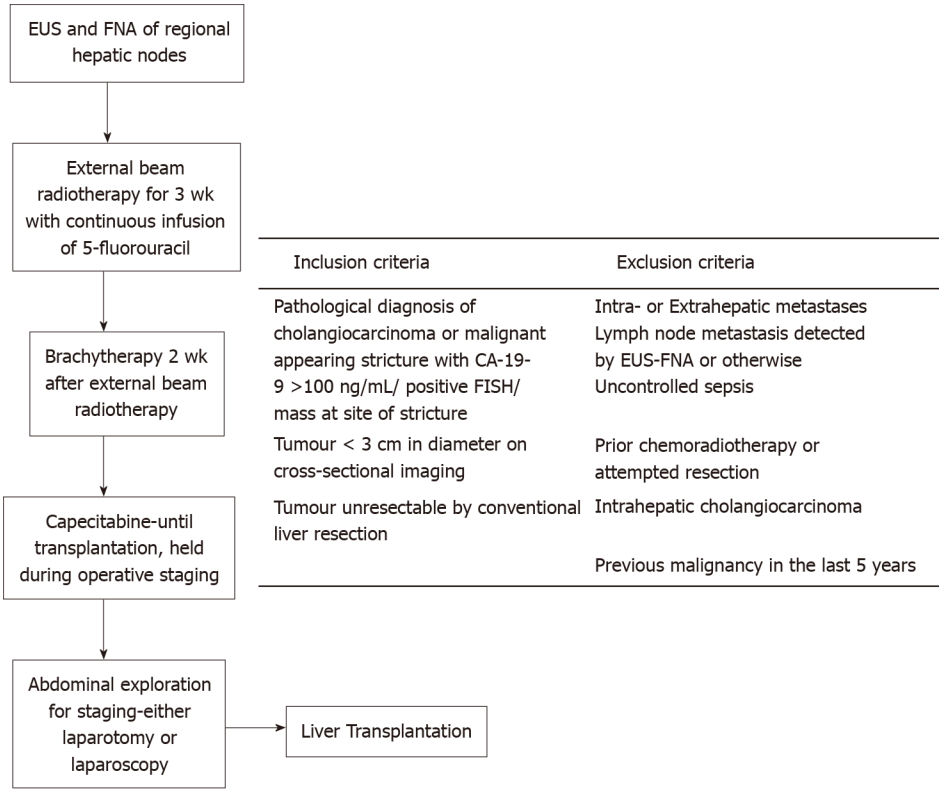
LIVER TRANSPLANTATION FOR CHOLANGIOCARCINOMA

These advances combined with use of newer cyclosporine and steroid immunosuppression allowed post-operative survival to increase significantly by the early 1980s and the Colorado group reported 1-year survival in excess of 70% during this time. This allowed meaningful evaluation of the oncological benefits of LT for the first time with postoperative recurrence rates and OS with cancer specific mortality. Of the 6 patients transplanted for unresectable CCA who survived more than 1 mo postoperatively, only 3 (50%) had recurrence free survival at 1 year. Those who did have recurrence all succumbed to disease at 1 year. The survival for those without recurrence at 1 year, ranged from 20 to 54 mo[21]. Poor survival for LT in CCA continued until the early 1990s, with 5-year overall, at less than 17% and recurrence greater than 50% in published series at the time[22-24].

The reasons for poor oncological outcomes despite optimisation of the LT procedure and immunosuppression protocols are in the main, twofold: Poor patient selection in early series, with presence of extrahepatic disease and utilisation for all anatomical subtypes of CCA, and absence of any neoadjuvant or adjuvant therapies to prevent recurrence.

This led to the development of neoadjuvant chemoradiotherapy and pre-transplant exploratory laparotomy protocols by the University of Nebraska[25] and Mayo Clinic in 1988 and 1993 respectively for phCCA only. The latter's results, initially published in 2000, showed of the 8 patients transplanted with follow-up > 12 mo, all were alive at their last follow-up (median 44 mo, range 17 to 83 mo) and only one patient developed a recurrence[26]. A subsequent study with 28 patients showed a 5-year actuarial OS rate of 82% with only 4 (14.2%) developing recurrence at 23 to 63 mo post-LT[27].

These impressive results have led to the adoption of the Mayo Clinic protocol more widely (Figure 2). In brief, patients with early stage hilar CCA on imaging (< 3 cm lesions, with no metastasis or lymph node involvement) who are technically unresectable undergo endoscopic ultrasound and fine needle



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Figure 2 Mayo clinic protocol for liver transplantation in hilar cholangiocarcinoma. EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; CA-19-9: Carbohydrate antigen 19-9; FISH: Fluorescence in situ hybridization.

aspiration of regional lymph nodes. Those with negative lymph nodes are given chemoradiotherapy for 5 wk and undergo laparoscopic or open abdominal exploration to exclude metastasis and examine regional lymph nodes prior to LT[27,28].

Current status of liver transplantation

There is a global discrepancy between donor livers availability and the number of patients on the liver transplant waiting list. Hence, there is understandably a clinical reticence in terms of utility to adopt LT for CCA given the historical poor prognosis when established benign and malignant indications for LT have proven significant benefit for this limited resource[29]. Established indications for LT include predominantly benign, chronic liver disease such as alcoholic and non-alcoholic fatty liver disease; autoimmune and metabolic pathologies such as primary sclerosing cholangitis (PSC) and Wilson's disease respectively and viral hepatitis. Paracetamol and other overdoses remain important acute causes of liver failure in Western populations which are also indications for LT. Among liver malignancies, the most widely established indication for LT is HCC, in which LT is only performed for early stage disease as outlined in the Milan Criteria[29]. Contemporarily 1- and 5-year OS for these indications following LT approach 95% and 85%, respectively[30]. New indications for LT will have to show comparable outcomes to the established indications to justify investment and allocation of donor livers for these purposes.

Following the publication of favourable results by the Mayo Clinic, with results similar to benign indications for LT, the United Network for Organ Sharing in United States, granted an exception to the Model for End Stage Liver Disease (MELD) used to prioritise those on the waiting list for LT. This exception allowed patients who had completed neoadjuvant therapy and had favourable operative staging to be added on the waiting list for LT with a MELD score equivalent to that of a patient with stage 1 HCC (as an established indication for LT)[31]. This improved access to LT for patients with phCCA and influenced the adoption of LT more widely with use of the MELD score and derivative scores internationally for LT allocations[29]. Further considerations of transplant allocation include the presence of underlying cirrhosis and greater degree of decompensation which increase the MELD score and may influence waiting time for transplantation but may also influence OS and recurrence rates when transplant is done for CCA.

The quality of the donor liver and whether it was from a live or deceased donor also has an impact on postoperative outcomes. Living donor LT may offer shorter waiting times, but is associated with higher rates of biliary complications (34% *vs* 17%, $P < 0.001$)[32]. Extended criteria donors with advanced age, steatosis or donors after circulatory death (DCD)[33] may increase the pool of organs but may be

associated with higher graft failure rates of 27.3% at 3-year with DCD livers *vs* 18.2% with donation after brainstem death[34]. Normothermic machine perfusion is another new technique which allows evaluation of graft function preoperatively and may further expand the donor pool and improve outcomes by selecting grafts with more objectively proven function[35].

PERIHILAR CHOLANGIOCARCINOMA

The Mayo Clinic group published their series of LT for phCCA from the inception of their protocol in 1993 to 2019. Of 376 patients included into the protocol, 234 (62%) ultimately proceeded to LT. The reasons for not proceeding to LT were primarily due to progression of disease prior to LT or metastasis found on staging surgery. OS at 5 years [Kaplan-Meier (KM)] was 68% +/- 3% and at 10 years was 60% +/- 4%[36]. Other long-term follow-up and larger series have validated the Mayo Clinic protocol for LT in phCCA. A multi-centre retrospective study from the United States of patients undergoing LT for phCCA, with any neoadjuvant chemo- or radiotherapy reported on 287 patients. Two hundred and fourteen (74%) proceeded to LT. Actuarial OS and disease-free survival (DFS) were 78% and 80% respectively at a median follow-up of 2.5 years. KM 5-year DFS was 65% [95% confidence interval (CI) 57%-73%] with 5-year OS at 53% (95%CI: 46%-60%)[37]. Multicentre series in the United Kingdom[37], Spain[38] and Germany[39] showed poorer results, likely as neoadjuvant protocols were not adopted at the time. Hidalgo *et al*[37] in the United Kingdom reported 5-year actuarial OS of 20%, Robles and colleagues 5-year OS of 30% in Spain and 31% in Germany pre-1998. Post-1998, when neoadjuvant chemotherapy was adopted in German protocols, 5-year OS after LT increased to 48%. A recent meta-analysis of retrospective series published before 2019 reported on 428 patients who underwent LT for phCCA and demonstrated pooled 5-year OS without neoadjuvant therapy of 31.6% (95%CI: 23.1%-41.7%) whilst with neoadjuvant therapy OS was 51.7% (95%CI: 33.8%-69.4%). Three years recurrence rates were 51.7% (95%CI: 33.8%-69.4%) without neoadjuvant therapy *vs* 24.1% (95%CI: 17.9%-30.9%), highlighting the importance of neoadjuvant regimes[40].

Comparison of LT to resection with curative intent for phCCA has shown a trend in favour of LT, albeit with high heterogeneity between studies. Ethun *et al*[14] performed a registry-based study of patients with phCCA at 3 United States centres, who underwent either curative intent liver resection or LT. Patients undergoing LT had neoadjuvant regimens similar to the Mayo protocol. One hundred ninety-one patients underwent curative intent resection (without neoadjuvant therapies), whilst 46 underwent LT. Five-year KM OS was 64% in the LT *vs* 18% for the resection group ($P < 0.001$). There was no significant difference in actuarial recurrence at a median follow-up of 23 mo, with 24% *vs* 37% ($P = 0.19$) for LT and resection groups respectively. Twenty nine percent of the patients undergoing LT had no residual disease on explant, suggesting complete response to neoadjuvant therapy. The authors also conducted an intention-to-treat analysis of patients who were enrolled in the transplant protocol and patients who were planned for curative intent resection or who had attempted curative intent resection. Five-year OS was still higher in the LT group at 53% *vs* 17% ($P < 0.001$) for the resection group[14].

Moris and colleagues performed a meta-analysis of studies with patients with locally advanced phCCA comparing LT and resection with curative intent. Local staging and nodal involvement were similar in both groups. There was a higher rate of R0 resection in patients with LT, 92.2% *vs* 73.3% [Risk Ratio (RR) 1.17, 95%CI: 1.03-1.33, $I^2 = 46\%$]. They also reported a higher 3-year OS with a lower hazard ratio (HR) of death (HR 0.61, 95%CI: 0.4-0.93, $I^2 = 39\%$). At 5 years, there was a trend towards increased OS for LT, but this was not significant (HR 0.67, 95%CI: 0.44-1.02, $I^2 = 54\%$)[41].

PSC

PSC is an important subgroup of patients undergoing LT in CCA. PSC is a significant risk factor for development of CCA and is in itself an indication for LT due to progressive liver dysfunction. PSC is associated with a higher rate of multifocal CCA precluding oncological resection or necessitating more extensive resection. Further, the poor quality of the liver parenchyma due to the disease may mean that the regenerative capacity of the liver is reduced which in turn limits the extent of resection possible. These factors typically preclude curative liver resection, even for small, localised lesions[43]. This prompted consideration for the alternative approach of LT for CCA, in particular phCCA, to mitigate the limitations of standard liver resection, and is one of the accepted inclusion criteria for the Mayo Clinic protocol[27]. Current data are clearly in favour of a protocolised approach with neoadjuvant chemoradiation followed by LT in PSC-associated phCCA, as the optimal treatment strategy, with superior outcomes when compared with de novo phCCA[42-44]. The Mayo Clinic series found a significantly higher 5- and 10-year survival in patients with PSC after LT, 74% and 67% *vs* 58% and 47% respectively in patients with de novo CCA[35]. The meta-analysis by Cambridge *et al*[40] also supports that in unresectable phCCA, combined neoadjuvant chemoradiation followed by LT confers long-term survival in highly selected patients able to complete the protocol with the most favourable outcomes observed in PSC patients[43]. Despite the lack of direct evidence, it has been hypothesized that the significantly better outcomes in PSC patients could be at least in part explained by a possible higher responsiveness to radiation therapy for phCCA arising on a background of PSC[35,42,45].

INTRAHEPATIC CHOLANGIOCARCINOMA

While phCCA is now increasingly accepted as an indication for LT using the Mayo Protocol, iCCA is still largely considered a contraindication[30]. Although early attempts at LT for CCA did not distinguish between subtypes in many cases and consequently iCCAs were transplanted[21] the outcomes remained poor until the advent of neoadjuvant protocols, such as that of the Mayo Clinic which are only applied to phCCA. Most literature on LT, therefore, focuses on incidentally found iCCA on explant, in patients who were transplanted for presumed HCC, which does not typically undergo neoadjuvant systemic therapy prior to transplant but rather only locoregional therapy such as radiofrequency ablation. There remains a lack of evidence to support such protocols for iCCA, partly because iCCA is the least common subtype of CCA (10% *vs* phCCA which represents 50%[46]) but also because of its distinct biological characteristics compared to other forms of CCA, with some being defined as mixed hepatocellular and intrahepatic cholangiocarcinoma[2].

Krasnodębski *et al*[47] provided one of the oldest series of 8 patients found on explant to have iCCA, having undergone LT without neoadjuvant therapies, from 1994-2019. Five-year OS and DFS were 25% and 28.6% respectively[47]. Lee *et al*[48] reported on their experience of LTs with pre-transplant diagnoses of HCC who were found to have iCCA on explant pathology. The majority of these patients also underwent neoadjuvant locoregional therapies such as radiofrequency ablation or transarterial chemoembolization. Seventeen cases of iCCA were identified from 1998-2018 and had 5-year actuarial OS of 51.9% and recurrence rate of 29.4%. This compared to the patients with HCC transplanted contemporaneously with OS of 71.5% and recurrence rate of 10.8%. Mixed hepatocellular and CCA LTs had a similar OS to iCCA at 55.0% but a significantly higher recurrence rate of 40.7%[48]. It was thought that perhaps the poor results for iCCA were due to poor patient selection as with phCCA and HCC. The latter is established as an indication for LT, but widely governed by the Milan criteria which restrict transplantation to early-stage tumours[49].

Sapisochin and colleagues performed a multicentre retrospective cohort study in Spain comparing incidentally found iCCA/mixed HCC/iCCAs on LT explant and LT for HCC. They found a significant decrease in survival in patients with pure iCCA compared to HCC (5-year actuarial OS 51% *vs* 93% respectively, $P < 0.001$), however mixed HCC/iCCAs had no significant difference in 5-year actuarial OS at 86% ($P = 0.9$). Subgroup analysis of patients with uninodular tumours < 2 cm in size, showed that of 12 patients with mixed HCC/iCCA ($n = 5$) and pure iCCA ($n = 7$), 5-year actuarial OS was 62% *vs* 80% in the HCC group ($P = 0.4$), implying no significant difference in survival. This finding may have been due to the low numbers of patients however[50].

A follow-up international multicentre retrospective series evaluating LT in early stage iCCA with unresectable unifocal tumours < 2 cm in size was conducted. Five-year actuarial OS in 14 patients with early stage iCCA was 65% *vs* 45% in those with advanced disease. KM recurrence rates were 18% at 5 years for early tumours compared to 61% for advanced tumours[51]. These figures compare favourably to curative intent liver resections with negative margins in iCCA with 5-year OS in the range of 30%-40% for all sizes of tumour. Early stage tumours < 2 cm in size can have near 100% survival at 5 years with negative margin resections. However, recurrence remains high with 5-year recurrence as high as 80% in the literature[52,53]. Hue *et al*[54] also did not find any significant differences in survival or recurrence with resection or LT for iCCA in their propensity matched registry-based study in the United States[54].

Studies which include neoadjuvant therapy prior to LT similar to the Mayo Protocol are limited to small case series. Lunsford *et al*[55] evaluated 12 patients with non-metastatic iCCA > 2 cm in size who were unresectable. They underwent neoadjuvant gemcitabine and platinum-based chemotherapy for 6 mo, and those who showed stable disease or regression proceeded to transplant. Six (50%) proceeded to LT and 5-year KM OS was 83.3% (95%CI: 27.3%-97.5%). Three patients developed recurrent disease at a median of 7.6 mo[56]. Wong *et al*[57] performed a similar study with the addition of transarterial chemoembolization and pre-transplant operative staging. Of 18 patients who started the neoadjuvant therapies only 5 (27.8) proceeded to transplant. Follow-up was limited to 1 year, actuarial OS was 80% (the single death was due to tumour recurrence) and recurrence developed in 2 patients (40%)[56].

A meta-analysis combining the above studies and others found a pooled 5-year OS of 42% (95%CI: 29%-55%) and 5-year DFS of 49% (95%CI: 41%-57%). However, the studies were all clinically heterogeneous with different preoperative protocols, different stages of tumours, degrees of cirrhosis in the background liver and used a variety of donor grafts. There was also statistically significant heterogeneity in all the meta-analyses with I^2 values being significant at the < 0.01 Level[57].

Interpretation of all the above results is difficult given significant clinical differences in each study. But overall, it can be concluded that while LT can improve outcomes compared to palliative therapy for unresectable iCCA, the outcomes compared to transplant for other indications including phCCA are poorer at present, although evidence is limited. Very early stage unresectable tumours < 2 cm in size appear to have excellent prognosis with LT, with improved outcomes compared to even curative intent resection for iCCA. More evidence is needed, however, there is increasing opinion that unresectable iCCA may become an extended indication for LT in the same way as phCCA has. In 2020, the European Network for the Study of CCA endorsed the value of this modality as a potentially curative option for iCCA in a consensus statement, and recommended it should be considered especially in patients with

very early stage unresectable tumours (≤ 2 cm) and concomitant cirrhosis[2].

A summary of key series of liver transplantation for phCCA and iCCA is presented in Table 1.

DISTAL CHOLANGIOCARCINOMA

phCCAs may extend down into the distal bile duct and it is established with the Mayo Protocol to potentially treat these with combined LT and pancreatoduodenectomy and many of the studies discussed above incorporate this either as a planned procedure or due to intraoperative findings of tumour extension[40]. Literature on LT in distal CCA (dCCA) is limited as these tumours are less likely to involve the liver and can be completely excised with pancreatoduodenectomy or bile duct excision with reconstruction depending on the site of the tumour. Extension into the liver or hilum would typically render the disease unresectable. Attempts at hepatopancreatoduodenectomy for these patients have been technically challenging with high rates of perioperative morbidity and mortality at up to 97.4% and 26% respectively in recent series. Five-year OS has ranged from 17.9%-49.2%[58]. Total hepatectomy, LT and pancreatoduodenectomy may have potential to be a better alternative therapy in this population. Case reports are sparse as dCCA is commonly an incidental finding in the context of LT for phCCA. Sutcliffe *et al*[59] report two patients at King's College Hospital, United Kingdom who underwent LT for PSC but were found to incidentally have dCCA on the bile duct explant. They then underwent pancreatoduodenectomy 2 mo post-transplant. One patient died due to other causes with no evidence of recurrent disease over 5 years later. The other patient died 5 mo after pancreatoduodenectomy due to recurrence[59].

Patients with PSC may also develop dCCA and require resection for this and also LT for their underlying liver disease. Stauffer *et al*[61] reported on 6 patients with dCCA and PSC who underwent both LT and classical Whipple's procedure. One patient survived to 58 mo of follow-up without any evidence of recurrence. Another patient, with an early T1 tumour survived to 52 mo of follow-up, with recurrence. All other patients developed recurrence and did not survive to 2 years[60].

DIRECTIONS FOR FUTURE RESEARCH

The literature at present consists mainly of retrospective case series of LT in CCA and predominantly in phCCA. The studies are heterogeneous in patient populations, in terms of staging of disease, preoperative neoadjuvant therapies, types of donor livers used and postoperative management and follow-up. Prospective multi-centre observational studies are needed for more robust evaluation of the Mayo Protocol and any modifications to the protocol should ideally be evaluated in a randomized trial. Unfortunately, due to the many confounding variables involved in LT and CCA and the rarity of suitable cases in general, recruitment to such trials with restrictive inclusion criteria to keep populations homogenous will no doubt be challenging.

The TRANSPHILL trial in France, has been ongoing since 2014 and aimed to recruit 54 patients with resectable phCCA for randomisation to either curative intent resection or LT. The primary outcome is 5-year OS, and 3-year recurrence is a secondary outcome[61]. Results are eagerly awaited from this trial as of writing, however, if the positive results of Ethun *et al*[14] are confirmed, this would represent a true paradigm shift away from resection to LT in phCCA.

The development of international registries may allow more generalisable and homogenous research beyond simple case series in future, by increasing the number of cases presented and capturing all relevant variables systematically.

Adjuvant and neoadjuvant protocols

Addition of newer neoadjuvant therapies such as stereotactic body radiation therapy (SBRT) have shown promise in preventing disease progression with lower toxicity than traditional external beam radiotherapy and may be beneficial prior to LT[62]. Oncological therapy has advanced in the management of CCA with improvement of survival with combination of folinic acid, fluorouracil, and oxaliplatin (FOLFOX) as a second line chemotherapy agent[63] and with recent trial results showing a benefit for immunotherapies in CCA[64]. Both have potential as neoadjuvant or adjuvant therapies in CCA patients considered for LT. Finally, the BILCAP trial established capecitabine as an effective adjuvant agent after resection of CCA as the standard of care, by showing improved OS[65]. There have been no studies on adjuvant therapy after LT for CCA and this too may improve survival in this setting.

Further, immunosuppression is known to be a risk factor for the development of malignancy and recurrence. There has been little research into the optimum suppressive regimen to balance the risks of graft rejection *vs* risk of recurrence after LT for CCA. In LT for HCC, there has been some evidence that reduction of immunosuppression[66] and use of mechanistic target of rapamycin (mTOR) inhibitors of suppression has a beneficial effect on OS after recurrence[67]. A recent series of patients undergoing LT for iCCA and phCCA showed that a reduced immunosuppressive regimen after recurrence was

Table 1 Summary of key contemporary series of liver transplantation for cholangiocarcinoma

Ref.	Country	Design	Tumour anatomical subtype	Treatments (s)	Total patients (*n*)	Median follow-up/yr (range)	Outcomes (5-year)[1]		
							Overall survival (%)	Disease free survival (%)	Graft survival (%)
Azad *et al*[28], 2020	United States	Retrospective, single centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	De novo: 148, PSC: 228	-	De novo: 58 KM, PSC: 74 KM	De novo: 55 KM, PSC: 78 KM	-
Darwish Murad *et al* [36], 2012	United States	Retrospective, multi-centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	287	2.5 (0.1-17.8)	53 KM	65 KM	60 KM
Hidalgo *et al*[37], 2008	United Kingdom	Retrospective, single centre	Perihilar	LT	12	1.81	41 KM	-	-
Robles *et al*[38], 2004	Spain	Retrospective, multi-centre	Perihilar	LT	36	-	64	47	-
Ethun *et al*[14], 2018	United States	Retrospective, multi-centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	70	4.83 (0.025-10.6)	64 KM	24 KM	-
Krasnodębski *et al* [47], 2020	Poland	Retrospective, multi-centre	Intrahepatic	LT	8	30	25	28.6	-
Lee *et al*[48], 2018	United States	Retrospective, single centre	Intrahepatic	Neoadjuvant TARE/TACE/RFA and LT	17	Mean 4.2	51.9	70.6	-
Sapisochin *et al*[50], 2014	Spain	Retrospective, multi-centre	Intrahepatic	Neoadjuvant ethanol injection/TACE/RFA and LT	27	4.99 (0.97-11.9)	51	36	-
Sapisochin *et al*[51], 2016	International	Retrospective, multi-centre	Intrahepatic- early[1] and advanced	Neoadjuvant ethanol injection/TACE/RFA and LT	Early[1]-15, Advanced-33	Early-4.78, Advanced-2.06	Early-65, Advanced-45	Early-86.7, Advanced-48.5	-
Hue *et al*[54], 2021	United States	Retrospective, multi-centre	Intrahepatic	Neoadjuvant chemo-radiation and LT	74	3.9	33 KM	-	-
Lunsford *et al*[55], 2018	United States	Prospective, single centre	Intrahepatic	Neoadjuvant chemotherapy and LT	6	3 (2.42-4.25)	83.3	50	100
Wong *et al*[56], 2019	United States	Prospective, single centre	Intrahepatic	Neoadjuvant chemotherapy and TACE and LT	5	1.84	80	60	-

[1]Early tumours defined as < 2 cm in size.

PSC: Primary sclerosing cholangitis; KM: Kaplan-Meier; LT: Liver transplant; TARE: Trans-arterial radioembolization; TACE: Trans-arterial chemoembolization; RFA: Radiofrequency ablation.

significantly associated with survival with an increased odds ratio of survival at 4.2 (95%CI: 1.3-13.6; P = 0.02)[68].

Further advances in LT and perioperative care along with novel chemotherapeutic and biological agents may lead to further improved outcomes[69].

Establishment of LT as a treatment modality for CCA

For LT to become uniformly established as a treatment modality for CCA, in both iCCA and phCCA, consistent reports with 5-year survival exceeding 50% and in the range of established indications of LT are needed in each group. The supply of donor livers will need to be improved to match increased demand from this population of patients and technologies such as normothermic machine perfusion and policy changes such as opt-out organ donation[70] being more widely enacted are expected to increase this supply.

CONCLUSION

LT has emerged as an effective treatment option for CCA in suitably selected patients. Treatment of unresectable phCCA has been transformed by LT with all modern series approaching parity with outcomes with other indications for transplant. Early evidence shows that LT may offer even better survival than curative intent resection for early resectable hilar tumours and may render transplant as the treatment of choice for the disease. Evidence for LT in iCCA is limited, but has considerable potential, with comparable outcomes to perihilar tumours in early disease and might become an established treatment option for suitable patients. Further improvements in LT and postoperative management along with novel chemotherapeutic and biological agents may further improve the current outcomes. Larger, high quality studies are needed in each group of tumours.

FOOTNOTES

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MINIREVIEWS

Research progress on the mitochondrial mechanism of age-related non-alcoholic fatty liver

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide. Reduced activity and slower metabolism in the elderly affect the balance of lipid metabolism in the liver leading to the accumulation of lipids. This affects the mitochondrial respiratory chain and the efficiency of β-oxidation and induces the overproduction of reactive oxygen species. In addition, the dynamic balance of the mitochondria is disrupted during the ageing process, which inhibits its phagocytic function and further aggravates liver injury, leading to a higher incidence of NAFLD in the elderly population. The present study reviewed the manifestations, role and mechanism of mitochondrial dysfunction in the progression of NAFLD in the elderly. Based on the understanding of mitochondrial dysfunction and abnormal lipid metabolism, this study discusses the treatment strategies and the potential therapeutic targets for NAFLD, including lipid accumulation, antioxidation, mitophagy and liver-protecting drugs. The purpose is to provide new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

Key Words: Non-alcoholic fatty liver disease; aging; Lipid metabolism; Mitochondria; Autophagy; Reactive oxygen species

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Core Tip: The elderly are prone to a series of pathological damages such as liver fibrosis due to the decline of liver regeneration ability and immune response dysfunction. In addition, liver metabolism imbalance and mitochondrial dysregulation play a key role in the development of non-alcoholic fatty liver disease (NAFLD). And a treatment strategy for NAFLD was proposed in terms of abnormal lipid metabolism, mitophagy, and anti-oxidation, which provided new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

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INTRODUCTION

In the past several decades, ageing has become a research hot spot with the understanding of cancer and metabolic disorder-related diseases. The ageing liver is also becoming a public health problem[1]. Due to the decline in the regenerative ability of the liver and dysfunctions in the immune response, older people are more likely to suffer from non-alcoholic fatty liver disease (NAFLD), acute and chronic liver injury, liver fibrosis and other diseases. Studies have reported that the prevalence of NAFLD increases in the elderly, with a prevalence of < 30% in people under 40 years of age and > 50% in people over 60 years of age[2].

Currently, it is believed that the mechanism of development of NAFLD includes increased production of fat, increased dietary free fatty acid (FFA) levels, β-oxidative damage and dysfunction in very low density lipoprotein synthesis[3]. However, reduced activity and changes in diet structure lead to a continuous increase in body fat in the elderly. These factors lead to the accumulation of TGs in the liver and eventually cause age-related NAFLD[4]. Studies have reported that the accumulation of TG droplets in hepatocytes is not a harmful process in itself. On the contrary, it is considered an adaptive response to excessive lipid uptake or the production of fat[5], and this imbalance in TG synthesis and breakdown causes fatty degeneration of the liver[6]. In addition, the structural and functional changes in the mitochondria have been shown to be related to the pathogenesis of NAFLD. Ultramicroscopic analyses have demonstrated a disordered morphology of hepatocyte mitochondria in elderly patients with NAFLD, and the damage to the structure and function led to fatty degeneration of the liver and other injuries[3]. The changes in mitosis and fusion of mitochondria during ageing lead to the inhibition of mitochondrial phagocytosis[7]. Cell function can be affected further if the damaged mitochondria are not cleared in time[8].

Age-related diseases are the major challenge in the 21st century. Delaying ageing is a significant challenge that people want to overcome. Ageing has become a very serious risk factor. Ageing starts with molecular damage and eventually leads to the dysfunction of cells, tissues and organs[9]. Clinically, the core of biological ageing is the increased vulnerability to death. The structural and functional changes in the mitochondria have been proven to be related to the pathogenesis of NAFLD, the loss of mitochondrial DNA (mtDNA) in hepatocytes affects function, leading to hepatic steatosis and other injuries[10]. The present study reviewed the manifestations, role and mechanism of mitochondrial dysfunction in the progression of NAFLD in the elderly. In addition, the study discusses the treatment strategies for NAFLD based on the understanding of mitochondrial dysfunction and abnormal lipid metabolism to provide new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

AGE-RELATED NAFLD: THE ROLE OF LIPID METABOLISM

Lipid metabolism is an important and complex biochemical process in the human body. The liver has a strong ability to synthesize fat, but it cannot store fat. Fatty degeneration of the liver occurs when fat is not transported in time. Simultaneously, the body decomposes fat through the action of various enzymes to maintain the dynamic balance of lipid metabolism. An imbalance in lipid metabolism ensues when there is a problem in decomposition. Therefore, in the event of fatty liver-related diseases, the primary consideration is to identify dysfunctions in the lipid metabolism pathway[11].

Enzyme regulation of β-oxidation of fatty acids

Mitochondria ensure a continuous supply of energy and metabolites to the organism through the aerobic oxidation of fatty acids, and the transfer of fatty acids into the mitochondria for β-oxidation

needs to be regulated[12]. In the mitochondria, the rate of lipid oxidation is significantly related to the entry of long-chain fatty acids into the matrix as well as β-oxidation. The long-chain acyl-CoA synthetase catalyses the fatty acids to synthetize the acyl-coenzyme A. Considering that the inner mitochondrial membrane (IMM) is impermeable to coenzyme A, the exchange of coenzyme A and carnitine is essential. The enzyme carnitine palmitoyltransferase-1 (CPT-1) is located on the outer mitochondrial membrane (OMM) and catalyses the reversible transfer of acyl-coenzyme A groups (with a chain length of C12 to C18) to L-carnitine to form acylcarnitine esters[13]. This newly generated acylcarnitine is subsequently transferred into the mitochondrial matrix in exchange for free carnitine. This translocation step is mediated by carnitine-acylcarnitine translocase (gene name: *SLC25A20*). Once in the mitochondrial matrix, acyl groups are transferred back to coenzyme A *via* CPT2. Human CPT1 exists in three forms: Liver subtype (CPT1A), muscle subtype (CPT1B)[14] and cerebral subtype (CPT1C)[15]. CPT1A and CPT1B have similar functions but are expressed in different tissues. The deficiency of CPT1A is associated with increased levels of L-carnitine in plasma, which provides a clue for the diagnosis of CPT1A deficiency. At the cellular level, the lack of CPT1A activity in the liver leads to the inability to generate acylcarnitines that enter the mitochondria for oxidation. Therefore, CPT1 has become an important target in the regulation of mammalian lipid metabolism.

Abnormal lipid metabolism in the early stage of NAFLD induces oxidative stress, and mitochondrial dysfunction is also an important aspect of oxidative stress. Damage to the mtDNA is the main cause of mitochondrial structural damage. Gene damage occurs due to a direct attack on mtDNA by various lipid peroxidation products, resulting in the reduction of the synthesis of its encoded mitochondrial complex and ATP synthase. Gene damage directly affects mitochondrial function. Lipotoxicity-induced oxidative stress disorder reflects the close relationship between abnormal lipid metabolism and mitochondrial dysfunction (Figure 1).

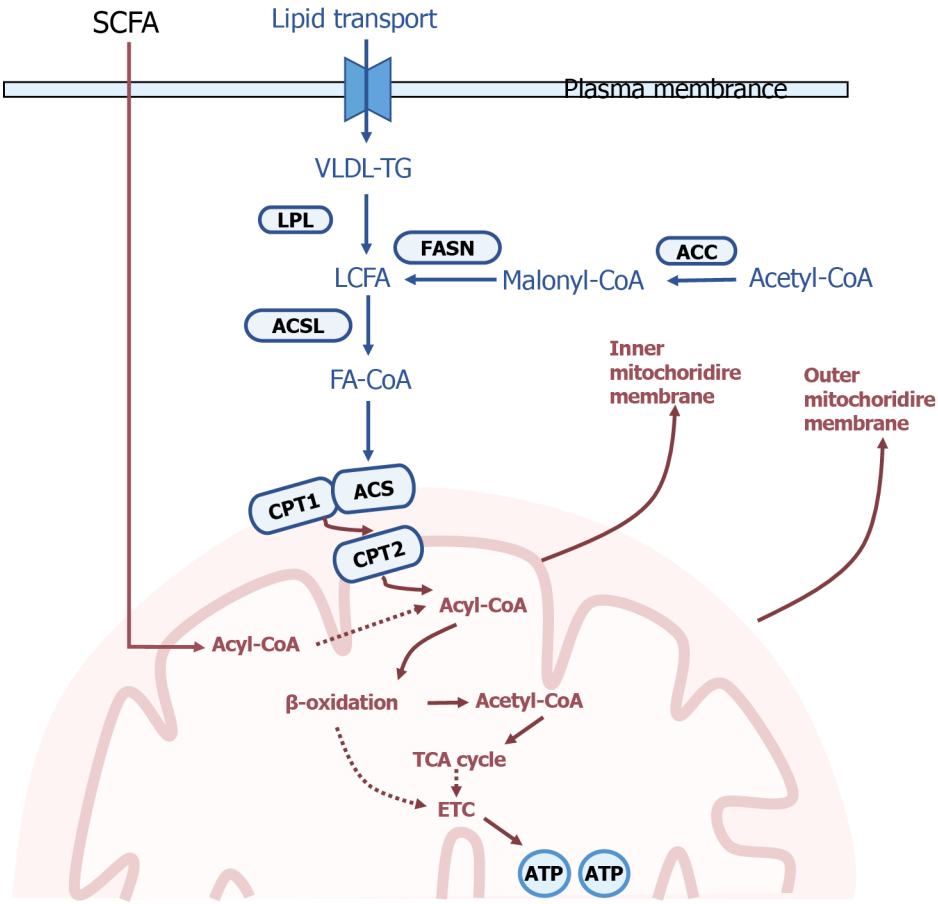
Regulation of nuclear receptor peroxisome proliferator-activated receptor for β-oxidation of fatty acids

In addition to various enzymes, nuclear receptors can also affect the β-oxidation of fatty acids[16]. Steatosis is one of the pathological characteristics of NAFLD, and fat accumulation leads to the activation of the nuclear receptor, peroxisome proliferator-activated receptors (PPARs)[17]. Studies conducted in 1990 revealed that drug experiments could cause the proliferation of liver peroxisomes, through which PPAR was discovered and later designated as PPARα (NR1C1). Subsequently, two additional PPARs were identified, namely, PPARβ/δ (NR1C2) and PPARγ (NR1C3). The expression organs and functions of the three PPARs are very different, PPARα is mainly expressed in the liver and brown adipose tissue[18]; PPARβ/δ is widely expressed in vivo, with relatively high expression in brain, stomach and intestine[19]; PPARγ expression is highest in white and brown adipose tissue[20]. PPARα is highly expressed in the liver, which is the main regulatory protein involved in hepatic β-oxidation, and is considered to be an important target for regulating dyslipidemia. Studies have shown that mitochondrial β-oxidation is significantly reduced in the liver of PPARα deficient mice[16]. Fatty liver can be improved by the activation of PPARα and the enhancement of hepatic fatty acid β-oxidation. Studies that investigated liver tissues from patients with NAFLD and non-alcoholic steatohepatitis (NASH)[21] reported that the expression of PPARα decreased with the progression of fibrosis. PPARα stimulants lead to decreased expression of substances associated with hepatic steatosis by enhancing mitochondrial β-oxidation. PPARα is involved in mitochondrial β-oxidation, with CPT1 as the key enzyme, allowing fatty acids to reach the mitochondrial matrix through the IMM and subsequently be metabolised. Abnormal PPARα expression can increase the levels of fatty acids and store them in the liver as triglycerides. PPARβ has been reported infrequently and has multiple functions and distributions, mainly being involved in wound healing. PPARγ is an important transcription factor for cell differentiation.

Regulation of the breakdown of lipid droplets

Lipid droplets (LDs) have a unique structure and exist in almost all cells. They are specialised organelles for storing lipids in cells. LDs provide energy for cells, aid in biofilm synthesis[22], and prevent FFA-induced lipotoxicity and its influx into toxic lipid species[23]. The accumulation of LDs in non-adipose tissue is a pathological feature of metabolic diseases such as obesity, diabetes and atherosclerosis[24]. In most cases, LDs exist in the form of an emulsion, which is the dispersed phase of water in oil in an aqueous solution. When large amounts of LDs are present, cells synthesize neutral lipids, which then form droplets dispersed in the aqueous phase[25]. The mechanism of the biogenesis of LDs remains unclear. The mechanisms underlying LDs biogenesis remain unknown, but studies suggest that LDs are simple structures surrounded by endoplasmic reticulum (ER)-derived phospholipid monolayers with dynamically changing proteome modifications on the surface[26]. A group of proteins, including seipin or lipid storage-inducing transmembrane proteins, are thought to be important for the formation of LDs. According to recent research, LDs are created de novo by neutral lipid deposition between ER leaflets and directed LD germination from the ER's outer leaflet into the cytoplasm[27].

LDs play a core role in cellular metabolism and homeostasis. Along with enhanced synthesis, decreased catabolism of LDs is another potential source of hepatic steatosis. There are two main



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Figure 1 Enzymatic regulation of fatty acid aerobic oxidation. SCFA: Short-chain-fatty acids; CPT1: Carnitine palmitoyltransferase-1; CPT2: Carnitine palmitoyltransferase-2; LCFA: Long-chain fatty acids; VLDL: Very low density lipoprotein; ACSL: Long-chain acyl-CoA synthetase; TCA: Tricarboxylic acid; ACS: Acyl-CoA synthetase; ACC: Acetyl-CoA carboxylase; ETC: Electronic respiratory chain; FASN: Fatty acid synthase.

pathways for the turnover of LDs in hepatocytes: conventional lipolysis and autophagy[28]. Lipolysis involves three key lipases, namely, hormone-sensitive lipase, adipose triglyceride lipase and monoacylglycerol lipase, which catalyse the rapid lipolysis of adipose tissue. In addition, some enzymes can directly interact with LDs to remove FAs from the triglyceride backbone stored in LDs one at a time. Furthermore, proteins also play an important role in the liver because the regulation of enzymes requires the participation of numerous hormones and growth factors through a variety of signal transduction pathways[29]. In addition to the above soluble lipases, hepatocytes also utilise the autophagy pathway to decompose LDs. Autophagy is mediated by lysosomes, and the formation of a special double-layer membrane vesicular structure during the process is called an autophagosome. In recent years, researchers have progressed in identifying the mechanism of other organelle-selective autophagy processes, such as mitochondrial phagocytosis and peroxidase phagocytosis[30]. Studies have found that lipid phagocytosis can be both non-selective and selective. Selective autophagy refers to the selective recognition and degradation of lipids, regulation of hepatocyte fat metabolism, and maintenance of intracellular lipid homeostasis.

Lipid metabolism in age-related NAFLD

Under normal circumstances, triglycerides are transported into the liver for lipid metabolism and are absorbed by hepatocytes. The main causes of NAFLD during ageing are an imbalance in hepatic metabolism, obesity, malnutrition and insulin resistance. However, there are few reports on the mechanism of development of age-related NAFLD, which needs further research in the future.

Obesity and metabolic abnormalities can be observed in elderly patients. The fat content in the body continues to increase with advancing age. Most of the fat exists in the form of triglycerides. The colour of the liver is closely related to the triglyceride content[31]. In addition to obesity, a low-quality diet and reduced activity in older patients can lead to malnutrition and muscle loss. Moreover, a low-quality diet can lead to an insufficient caloric supply and low protein levels, leading to an increase in the serum levels of FFAs. Furthermore, severe insulin resistance in skeletal muscles during ageing can induce the upregulation of the cholesterol regulatory element-binding protein 1, which inhibits β-oxidation and leads to fat deposition in the liver. Molecular substances related to NAFLD interfere with the cascade

reaction of the insulin signalling pathway and aggravate insulin resistance. Studies have also reported that compared with young individuals, glycogen synthesis in the muscles of elderly individuals decreases by 45%, the liver fat re-synthesis rate is twice higher, and the fasting blood triacylglycerol level is significantly higher, which increases by approximately three times after food[32].

ROLE OF MITOCHONDRIAL DYSREGULATION IN AGE-RELATED NAFLD

Mitochondria, which account for about one-fifth of the cell volume, are the organelles that mainly produce ATP in eukaryotic cells[33]. Almost all energy in the human body is generated by the tricarboxylic acid cycle and the electron transport chain of mitochondria, which is produced in the form of ATP. Mitochondria have inner and outer bilayers. The permeability of the inner membrane is lower than that of the outer membrane. The enzymes involved in the electron transport chain and ATP generation process are located in the inner membrane. An important feature of human ageing is a decrease in mitochondrial function in various tissues. Therefore, mitochondrial defects or dysregulation play a key role in ageing as well as diseases such as cancer[34].

Mitochondrial homeostasis

Mitochondria are dynamic organelles that continuously undergo fusion/division, and their homeostatic balance is essential for the maintenance of function. Many genes associated with mitochondrial homeostasis are human disease genes. Mitochondrial dynamics are regulated by two opposite processes, fusion and fission. The processes are essential to regulate mitochondrial morphology, number, function and sub-cellular distribution, as well as to maintain mitochondrial homeostasis to cope with stress[35]. Mitochondrial dynamics vary according to the developmental stage, age, cell type, environmental factors and genetic background. Mitochondrial homeostasis is affected during ageing. The structure of the mitochondria becomes abnormal with advancing age, biogenesis is reduced, and mtDNA mutation is increased[36].

During ageing, the changes in mitochondrial fission and fusion lead to the inhibition of mitochondrial phagocytosis, and further damage to cellular function occurs if the damaged mitochondria are not cleared in time. The process of mitochondrial fusion requires the inner and outer membrane proteins, optic atrophy 1 (OPA1) protein and mitotic fusion proteins (MFN1 and MFN2)[37]. Deletion of the mitotic fusion proteins blocks the fusion of the inner and outer membrane proteins (IMM and OMM), while deletion of OPA1 blocks the fusion of the IMM but not the OMM[38]. The genetic disappearance of OPA1 changes the morphology and activity of lysosomes, thus inducing the accumulation of autophagic substrates. Mitochondrial fission is mediated by several proteins, with mitochondrial motility-related proteins being central. When mitochondria divide, dynamin-related protein 1 (DRP1) is located in the proteins and factors on the OMM to form a ring structure. The ring is divided into two mitochondrial cristae through the GTP enzyme activity of DRP1. After cell division, DRP1 returns to the cytoplasm to participate in the next mitochondrial division[39].

Mitochondria maintain their morphology and function in cells through continuous fusion and division[40]. Studies have found that the mitochondria in the liver of mice with NAFLD become smaller and fragmented[41]. Steatosis and inflammatory reaction in the liver are aggravated in mice with a knockdown of the MFN2 gene that controls mitochondrial fusion, promoting the progression of liver fibrosis and liver cancer. Wang *et al*[42] reported that mice with NAFLD and hepatic mitochondrial DRP1 gene knockout had mitochondrial fission disorder, which directly promoted ER stress and hepatocyte death. In the pathological state of age-related NAFLD, oxidative stress in the liver aggravates mitochondrial functional and structural damage. The damage can manifest as an imbalance in the regulation of mitochondrial quality, such as the splitting of mitochondria, increased autophagy and fusion, and reduced biosynthesis, thereby affecting liver function and energy metabolism and promoting NAFLD[43,44].

Mitochondrial reactive oxygen species

The acellular production of reactive oxygen species (ROS) is an inevitable process. Under normal circumstances, oxygen metabolism generates an adequate amount of ROS required for detoxification. An excessive amount of toxins will lead to tissue damage and inflammation; however, cells have several defense systems to fight them. Most data indicate that oxidative damage increases in older adults. Some studies have reported that the antioxidant defense capacity decreases with age, while others have not reported any changes[45]. Compared with other rodents, naked mole rats, a long-lived rodent, have an increased amount of ROS. However, these rats also have an increased free radical scavenging ability, which does not decline with age[46]. Intracellular ROS may also play important roles in intracellular signaling, which may be beneficial to the ageing process. Therefore, in our opinion, ROS are not necessarily harmful, and the balance between ROS and scavenging may be the key.

It has been found that the mitochondrial respiratory chain and β-oxidation are gradually impaired in hepatocytes of NAFLD, which induces the generation of excess amounts of ROS[47]. Superoxide anions, hydroxyl radicals, peroxy radicals, and other non-radicals that can produce free radicals are all

members of the family of free radicals known as ROS[48]. The overproduction of ROS is related to oxidative damage to lipids, DNA and proteins[49,50]. According to the multiple parallel hit theory, oxidative stress is considered a major factor in liver injury and disease progression[51]. Oxidative stress has increasingly become one of the important pathological causes of the development of NAFLD, and it is the link between simple steatosis and symptoms of NASH[52].

Lipids are one of the main sources of mitochondrial ROS in NAFLD. Mitochondria can produce ROS during oxidative phosphorylation (OXPHOS), which can trigger mitochondrial dysfunction (such as DNA, proteins, lipids and other molecules) by interacting with mitochondria and cellular components [53]. Mitochondrial function and ROS production are considered important regulators of lifespan. Mitochondrial dysfunction leads to the overproduction of ROS. Oxidative damage may be involved in various pathological processes. At present, an imbalance in the mitochondrial redox state is considered the main cause of cell damage. The interaction between mitochondria and peroxisomes regulates metabolic and redox signaling pathways through the mitochondrial delivery system[54]. Mitochondrial ROS are considered an important factor in the development of liver disease. The production of mitochondrial ROS may lead to fatal cell damage due to the impairment of several bioenergetic responses involved in OXPHOS[55]. In addition, due to its proximity to ETC, mtDNA is extremely prone to oxidative damage, leading to DNA breakage and mutation[56].

Mitophagy

The understanding of the physiological role of autophagy in mammals has increased in the past decade. Autophagy is associated with many physiological processes[57]. The main function of autophagy is to degrade endogenous biomacromolecules and recycle cellular substances. There are three main forms of autophagy[58], namely, microautophagy, macroautophagy and chaperone-mediated autophagy. The difference lies in the mode of transportation. Autophagy has become a potential anti-ageing mechanism [59]. Autophagy declines with age and the expression of several key indicators in the autophagy pathway (such as ATG5 and ATG7) show a downward trend in the brain of older people[60]. Caloric restriction and exercise can enable autophagy to delay aging-associated degeneration[61].

The ageing of cellular mitochondria gradually makes it inefficient and potentially toxic. An acute injury can increase the permeability of the mitochondrial membrane, thus triggering apoptosis or necrosis. Autophagy inhibits inflammation through phagocytosis of dysfunctional or damaged mitochondria, preventing unnecessary cell loss[62]. Autophagy can not only regulate lipid metabolism and insulin resistance[63] but also degenerative diseases caused by the reduced expression of autophagy or mitophagy genes, including inflammation and cell population death due to lack of quality control. A growing body of evidence also shows that hepatic autophagy is impaired in NAFLD[64], thus confirming the theory that the combination of mitochondrial dysfunction and insufficient autophagy may lead to a variety of age-related diseases, and making autophagic flux a potential pharmacological target in NAFLD.

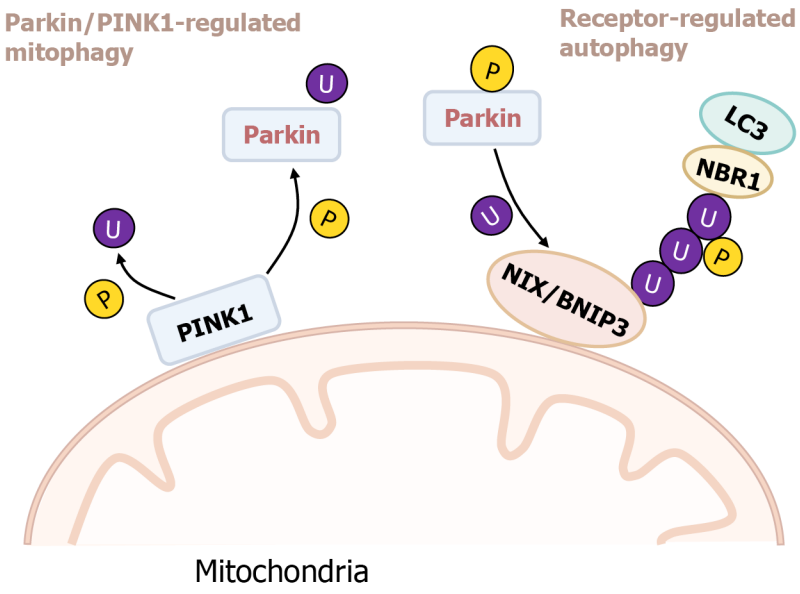
Presently, two main mechanisms are known to regulate mitochondrial autophagy, namely, mitophagy regulated by parkin/PINK1[65] and receptor-regulated autophagy[66]. Parkin is an E3 ubiquitin ligase located in the downstream. PINK1 is a serine/threonine kinase located in the upstream. Autophosphorylation of PINK1 is essential for ubiquitin recognition and can promote the translocation of phosphorylation for parkin from the cytosol to mitochondria. In the event of mitochondrial damage, the membrane potential decreases, leading to the accumulation of PINK1 in the OMM, attracting parkin to attach to mitochondria and causing further autophagy. In addition, Nip3-like protein X (NIX) and Bcl-219 kDa interacting protein (BNIP3) are outer membrane proteins associated with autophagy and apoptosis under hypoxia, which can act as mitochondrial autophagy receptors. Studies have confirmed that NIX can also participate in the process of parkin-dependent mitochondrial autophagy. It can be ubiquitinated as a substrate of parkin and recruit the LC3 adaptor protein NBR1 to target mitochondria to autophagosomes and induce mitochondrial autophagy (Figure 2)[67].

Mitochondrial dysfunction is characterised by an imbalance in mitochondrial homeostasis, ROS overproduction, autophagy abnormalities, respiratory chain disorders and mtDNA damage. A large number of studies have demonstrated structural and functional alterations in the mitochondria of hepatocytes in NAFLD (Figure 3)[68-70].

Mitochondria and gut microbiome

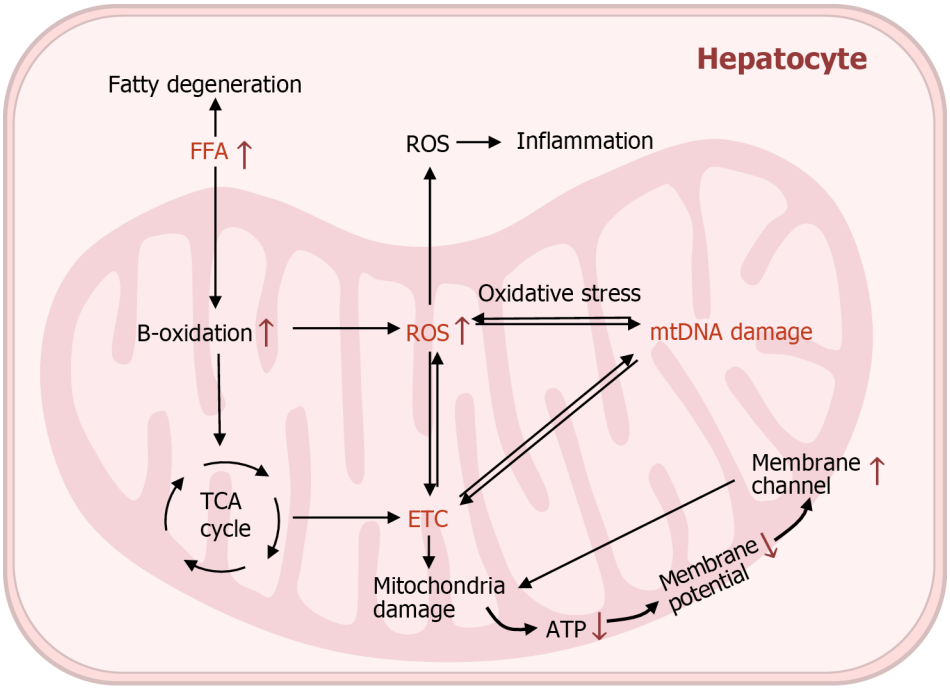
Appreciation of the role played by the gut microbiome has increased rapidly in recent years[71-73]. Comprehensive studies on the aetiology of NAFLD induced by excess nutrition and obesity have shown that changes in the gut microbiome are crucial in the development of NAFLD[74]. Dysregulation of gut flora has been shown to be an important factor in age-related pathology.

Mitochondria regulate intestinal function, including barrier defense of the intestinal epithelial cells [75-77]. Studies have shown that mitochondrial dysfunction in colonic epithelial cells induced by the use of dinitrophenol leads to dysfunction of the intestinal barrier[78], suggesting that mitochondrial dysfunction impairs the functional integrity of intestinal epithelial cells. MitoTEMPO, an antioxidant that targets mitochondria, inhibits these barrier defects, suggesting that mitochondrial stability is important for maintaining intestinal barrier function[71]. Unbalanced gut flora may contribute to the development and progression of multiple diseases. Recently, disorders of the intestinal flora have been



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Figure 2 Mitochondrial depolarization activates the PINK1/Parkin pathway to mediate mitophagy. NIX: Nip3-like protein X; BNIP3: Bcl-219 kDa interacting protein.



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Figure 3 Mitochondrial depolarization activates the PINK1/Parkin pathway to mediate mitophagy. FFA: Free fatty acid; ROS: Reactive oxygen species; TCA: Tricarboxylic acid; mtDNA: Mitochondrial DNA; ETC: Electronic respiratory chain.

implicated in liver diseases, such as hepatitis, cirrhosis and NAFLD[74].

THERAPIES TARGETING MITOCHONDRIAL FUNCTION IN AGE-RELATED NAFLD

Currently, there is no approved drug treatment for NAFLD. Hannah *et al*[79] found that a 3%-5% weight loss in patients with NAFLD slowed the progression of the condition. Although lifestyle changes through appropriate diet and exercise have been shown to be beneficial, most patients find it difficult to achieve and maintain the same. Therefore, identifying effective therapeutic drugs is an active area of

research. Clinically, the pharmacological agents for NAFLD mainly include lipid-lowering drugs, liver-protecting drugs, antioxidants, autophagy inhibitors and insulin sensitizers.

PPAR ligand therapy

The disorder of fat metabolism in the human body is one of the important reasons for the development of NAFLD. Aiming at the accumulation of liver fat, the PPAR ligand can be considered a possible therapeutic agent for NAFLD. Regulating lipid metabolism and reducing blood lipids is also a popular approach for the treatment of NAFLD[80]. The latest evidence from preclinical and clinical studies confirms the potential of PPAR ligands to treat this series of liver diseases. For example, elafibranor, a PPAR agonist[81], can increase mitochondrial fatty acid oxidation and oxidative phosphorylation and reduce the flow of fatty acids from adipose tissue to the liver. Presently, a phase III clinical trial (NCT02704403) has been completed.

Hepatoprotective drugs

Hepatoprotective drugs are commonly used as adjuvant in the treatment of NAFLD, which can not only protect the liver but also resist oxidation, inflammation and fibrosis and prevent the probability of malignant transformation of liver disease. Common hepatoprotective drugs include polyene phosphatidylcholine and obeticholic acid (OCA). Polyene phosphatidylcholine has anti-oxidative and anti-inflammatory effects that aid in reducing liver cell damage and even apoptosis and can effectively target the pathological symptoms caused by NAFLD. The effect of its combination with metformin in the treatment of patients with NAFLD and NASH is significantly better than that of monotherapy[82]. OCA is a farnesoid X receptor agonist. As a synthetic lipophilic bile acid, OCA can effectively reduce hepatic steatosis in mice[83].

Antioxidant drugs for inhibiting hepatocyte apoptosis

Vitamin E is an antioxidant and has been proven one of the more effective drugs for the treatment of NAFLD. Clinical studies have reported significant improvement in blood lipid biochemical indicators and histopathological changes in the liver after oral administration of VE at 800 IU/d for 96 wk. The results indicated that VE was beneficial for the treatment of NAFLD and NASH[84]. Sanyal *et al*[85] found that oral VE at 800 IU/d for 2 years could improve hepatic steatosis and inflammatory injury. Lv *et al*[86] hypothesised that the accumulation of ROS was the process of quiescent cells entering the cell cycle. Through experiments, they confirmed that the expression of cell cycle regulators P16 and P38 was related to the production of ROS, which provided a feasible therapeutic target for scavenging ROS and improving NAFLD.

Improve mitophagy

Improving mitophagy has been a hot topic in the treatment of NAFLD in recent years. Wu *et al*[87] found that a high-fat diet induced an increase in the expression of a mixed series of protein kinase-like domains (MLKL). MLKL can combine with liver mitochondria to stimulate the production of ROS and transfer it to the autophagosome membrane to inhibit the autophagy of injured cells and exacerbate liver injury. Therefore, MLKL-knockout models can improve the autophagy defect of injured hepatocytes and alleviate the progression of NAFLD. Studies have suggested the feasibility of inducing autophagy in the treatment of NAFLD and the application of related targets. However, excessive induction of autophagy may aggravate liver injury; therefore, maintaining the balance between them is the key to treating NAFLD.

CONCLUSION

In summary, as the central link of energy metabolism, mitochondria play an important role in maintaining the normal structure and function of age-related NAFLD. There is a concomitant and progressive relationship in the process of age-related NAFLD, from fatty degeneration to a series of pathological lesions, such as inflammation and liver fibrosis. However, whether a mitochondrial disorder of hepatocytes is the cause of the progression of age-related NAFLD or the result of abnormal lipid metabolism and lipotoxicity cannot be fully confirmed[88]. The occurrence of mitochondrial dysfunction is closely related to the disorders of dynamic balance, mtDNA deletion, excessive production of ROS, abnormal mitophagy and abnormal fatty acid oxidation. A better understanding of the mechanism of mitochondrial dysfunction will be helpful to develop new therapeutic strategies for NAFLD. In the next few years, the availability of therapeutic options for NAFLD is expected to curb the rising trend of related diseases. More rigorous experiments should be designed to validate the results and develop more targeted drugs that regulate mitochondrial function to treat NAFLD, thus achieving the transformation from basic theory to clinical practice.

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ORIGINAL ARTICLE

Case Control Study

Gut microbiota predicts the diagnosis of celiac disease in Saudi children

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Abstract

BACKGROUND

Celiac disease (CeD) is a multisystem immune-mediated multifactorial condition strongly associated with the intestinal microbiota.

AIM

To evaluate the predictive power of the gut microbiota in the diagnosis of CeD and to search for important taxa that may help to distinguish CeD patients from controls.

METHODS

Microbial DNA from bacteria, viruses, and fungi, was isolated from mucosal and fecal samples of 40 children with CeD and 39 controls. All samples were sequenced using the HiSeq platform, the data were analyzed, and abundance and diversities were assessed. For this analysis, the predictive power of the microbiota was evaluated by calculating the area under the curve (AUC) using data for the entire microbiome. The Kruskal-Wallis test was used to evaluate the significance of the difference between AUCs. The Boruta logarithm, a wrapper built around the random forest classification algorithm, was used to identify important bacterial biomarkers for CeD.

RESULTS

In fecal samples, AUCs for bacterial, viral, and fungal microbiota were 52%, 58%, and 67.7% respectively, suggesting weak performance in predicting CeD. However, the combination of fecal bacteria and viruses showed a higher AUC of 81.8 %, indicating stronger predictive power in the diagnosis of CeD. In mucosal samples, AUCs for bacterial, viral, and fungal microbiota were 81.2%, 58.6%, and 35%, respectively, indicating that mucosal bacteria alone had the highest predictive power. Two bacteria, *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, in fecal samples and one virus, *Human_endogenous _retrovirus_K*, in mucosal samples are predicted to be "important" biomarkers, differentiating

celiac from nonceliac disease groups. *Bacteroides intestinalis* is known to degrade complex arabinoxylans and xylan which have a protective role in the intestinal mucosa. Similarly, several *Burkholderiales* species have been reported to produce peptidases that hydrolyze gluten peptides, with the potential to reduce the gluten content of food. Finally, a role for *Human_endogenous _retrovirus_K* in immune-mediated disease such as CeD has been reported.

CONCLUSION

The excellent predictive power of the combination of the fecal bacterial and viral microbiota with mucosal bacteria alone indicates a potential role in the diagnosis of difficult cases of CeD. *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, which were found to be deficient in CeD, have a potential protective role in the development of prophylactic modalities. Further studies on the role of the microbiota in general and *Human_endogenous _retrovirus_K* in particular are needed.

Key Words: Celiac disease; Microbial signature; Children; Saudi Arabia

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Core Tip: Celiac disease (CeD) is known to be associated with the microbiota. In this study, the combination of bacterial and viral taxa in stools and mucosal bacterial taxa were the strongest predictors of celiac disease. In addition, we report important bacterial markers, namely, *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, which were reduced in children with CeD, suggesting a protective role in children with CeD.

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INTRODUCTION

Celiac disease (CeD) is an immune-mediated condition with multisystem clinical expression[1,2]. The disease is distributed worldwide, and the incidence is increasing. The global seroprevalence and biopsy-proven prevalence are estimated to be 1.4% and 0.7%, respectively[3]. In the Kingdom of Saudi Arabia (KSA), a seroprevalence between 1.5% and 3% and a biopsy-proven prevalence of 1% are some of the highest observed rates in the world[4,5]. The pathogenesis of CeD is multifactorial, requiring genetic susceptibility in the form of human leukocyte antigen DQ2 and DQ8 genotypes and exposure to gluten-containing food[6]. In the KSA, high prevalence of genetic susceptibility of 47% has been reported[7]. Although genetic susceptibility and exposure to gluten-containing food are necessary, not all genetically susceptible individuals develop CeD. Moreover, in some cases, CeD develops later in life after many years of gluten ingestion, indicating that other factors may be important in loss of tolerance to gluten and development of clinical disease[8,9]. Microbial dysbiosis associated with CeD is thought to be one of the important environmental factors contributing to loss of tolerance to gluten and thereby playing a role in pathogenesis of CeD[10,11]. However, identification of microbial markers and the predictive power of microbial dysbiosis in CeD have rarely been reported[12]. Therefore, the objectives of this study were to determine the predictive power of the gut microbial community in the diagnosis of CeD and to search for taxa that may be important in differentiating children with CeD from those without CeD.

MATERIALS AND METHODS

Shotgun metagenomic analysis of fecal and duodenal mucosal samples from children with new onset CeD was performed for bacteria, viruses, and fungi. Briefly, there were 40 children with CeD. Patients were eligible if they had confirmed CeD by standard criteria and not received antibiotics for at least 6 mo. They were enrolled as they presented to clinics. There were 39 non-CeD controls, including 20 school children who were clinically healthy who provided stool samples and 19 from whom tissue samples from the second part of the duodenum were collected during diagnostic endoscopy performed for clinical indications. The children with CeD and controls were enrolled in the study after consent/assent. All children were recruited from King Khaled University Hospital and King Fahad Medical City,

both institutions are in Riyadh (KSA). Microbial DNA was isolated and sequenced using the HiSeq platform. The results included abundance and diversity analyses for bacteria, viruses, and fungi that were recently reported[13-15].

For the purpose of this report, receiver operating characteristic (ROC) analysis with calculation of the area under the curve (AUC) was used to assess the predictive power of the gut microbiota in the diagnosis of CeD. ROC analysis and calculation of the AUC for discrimination using data regarding microbial communities, including bacteria, viruses, and fungi, in stool and mucosa were performed. Boruta analysis was used to identify important taxa that may differentiate children with CeD from non-CeD controls. In brief, the Boruta logarithm is a wrapper built around the random forest classification algorithm implemented in the R package random forest[16]. The Boruta process consists of assigning an 'importance' score to each variable and identifying a threshold above which the variables are deemed important and below which they are not. This process is repeated to establish reproducibility and robustness and therefore generates many 'importance' scores for each taxon. Species-level relative abundance data were used to generate shadow variables to predict taxa that may be important in distinguishing celiac from nonceliac groups[17]. Sensitivity and specificity were calculated based on the output from a random forest classifier[18]. Bioinformatics and statistical analyses were performed by specialists at Cosmos ID, United States (https://www.cosmosid.com/).

RESULTS

The areas under the curve for the bacterial, viral, and fungal microbiota in fecal samples were 52%, 58%, and 67.7%, respectively, suggesting poor performance for each, in predicting CeD. However, the combination of fecal bacteria and viruses revealed a higher AUC of 81.8 %, indicating a stronger predictive power for the diagnosis of CeD (Figure 1). Nevertheless, the difference between the AUC of the bacterial microbiome alone and the combined bacterial and viral microbiomes showed borderline significance (P = 0.05211).

The areas under the curve for the bacterial, viral, and fungal microbiota in mucosal samples were 81.2%, 58.6%, and 35%, respectively, indicating the the highest predictive power for mucosal bacteria alone (Figure 2). The difference in AUC between mucosal and fecal bacteria was significant (P = 0.01885).

The scores for the confirmed important variables are summarized in Table 1, including the mean, median, minimum, and maximum importance values. The 'Decision' column indicates 'Confirmed' for the microbiota above the threshold set by Boruta analysis. The microbiota including two bacteria (*Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*) and one virus (*Human_endogenous _retrovirus_K*), was confirmed to be important for distinguishing between CeD and non-CeD groups.

The results of the Boruta random forest algorithm analysis are illustrated in Figure 3. Two bacteria (*Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*) in fecal samples and one virus in mucosal samples (*Human_endogenous _retrovirus_K*) were predicted to be "important" for differentiating celiac from nonceliac disease groups. No fungal species were found to be important.

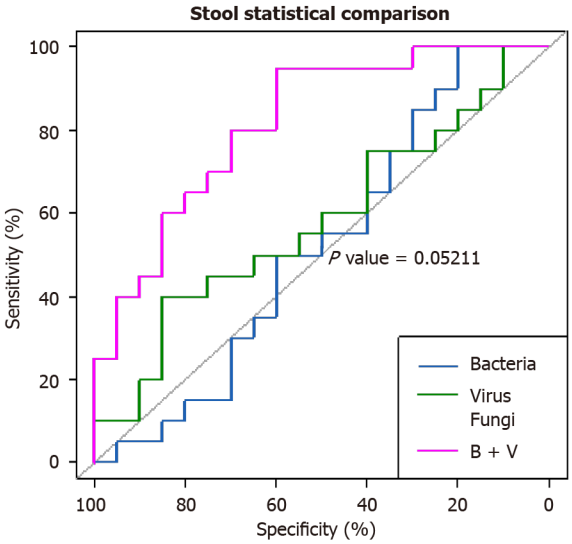
DISCUSSION

The role of the microbiota in predicting diseases in general has been largely reported. However, to our knowledge, the sensitivity and specificity of the gut microbiota in distinguishing CeD from non-CeD has not been reported thus far. In this study, the finding of a high AUC of the combination of bacteria and viruses in fecal samples (81.8%) indicates excellent predictive power with potential use in the diagnoses of difficult cases of CeD. Similarly, the significantly higher AUC for bacteria (81.2%) in the mucosal than in the fecal samples indicates stronger predictive power for mucosal bacteria (P = 0.01885). However, this was not surprising, as CeD is mainly a mucosal small bowel disease. These findings may have potential applications in the diagnosis of difficult cases of CeD.

Identification of the "important" specific microbiota in CeD in the form of *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* has not been previously reported. *Bacteroides intestinalis* belongs to the *Bacteroides* genus, members of which are known to degrade complex arabinoxylans and xylan from dietary fibers, including wheat, rye, oat, and barley[19]. These degradation products, including butyrate and ferulic acid, have been shown to have a protective role in the intestinal mucosa[20-22]. *Burkholderiales bacterium 1-1-47* is an unclassified bacterium belonging to the order *Burkholderiales*, class *Betaproteobacteria* and phylum *Proteobacteria*[23]. Several *Burkholderiales* species and *Burkholderia gladioli* in particular have been reported to produce peptidases that hydrolyze gluten peptides, with the potential to reduce the gluten content of food[24]. Accordingly, reports of decreased abundance of both *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in samples from children with CeD[13], indicate a potential protective role against the effects of gluten-containing grains.

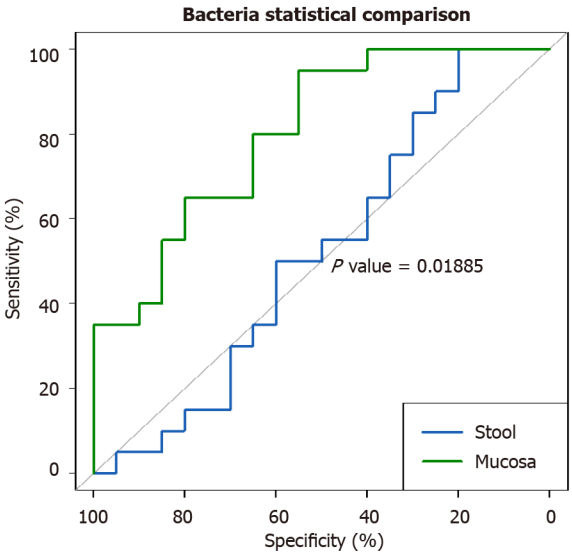
Table 1 Scores of important microbiota identified by Boruta analysis

Microbial species	Mean importance	Median importance	Minimum importance	Maximum importance	Decision
Bacteroides_intestinalis	6.92517709	7.70811062	1.25328634	9.93239532	Confirmed
Burkholderiales_bacterium_ 1_1_47	5.39952346	5.77233858	-1.307345	9.24744767	Confirmed
Human_endogenous _retrovirus_K	9.95761324	0.6340023	3.26946721	3.8621105	Confirmed



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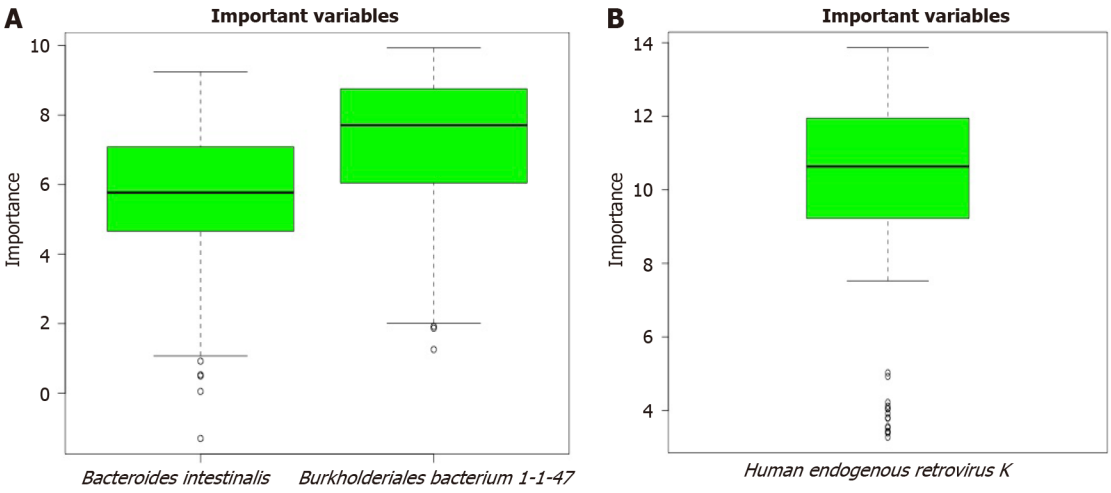
Figure 1 Comparative area under the curves of the fecal microbiota show that the combination of bacteria and viruses was the strongest predictor of celiac disease. However, the difference between the area under the curve of bacteria alone and combined bacteria and viruses was borderline significant (*P* = 0.05211). B + V: Bacteria plus viruses.



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Figure 2 Comparison between mucosal and fecal bacterial area under the curves shows that mucosal bacteria were significantly stronger predictors of celiac disease (*P* = 0.01885).

Confirmation of *Human_endogenous _retrovirus_K* virus as important in differentiating CeD from non-CeD groups is interesting. This group of viruses has been suggested to have a role in immunity and autoimmune disorders. They can contribute to host protection or to damage, suggesting a subtle balance between the persistence of human endogenous retroviruses expression and maintenance of a basal immune alert[25]. Although a recent study found increased expression in children with CeD[26], identi-



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Figure 3 The microbiota predicted important by Boruta random forest algorithm. These included two bacteria in fecal samples. A: *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*; B: One virus mucosal samples, *Human_endogenous _retrovirus_K*.

fication of *Human_endogenous _retrovirus_K* as important and the significantly reduced abundance in children with CeD[13], should prompt further investigation of the role of these viruses in children with CeD.

Study limitation

This study had a relatively small sample size. In addition, the non-CeD controls were not completely healthy although they do not have CeD as TTG-A, endoscopy, and duodenal tissue histopathology were normal. However, the relatively small size might be partially compensated for by the use of the shotgun metagenomic analysis. Since this is the first report on microbiota accuracy and identification of important bacteria and viruses, but not fungi, further studies with larger sample sizes are needed.

CONCLUSION

The high AUCs of mucosal bacteria and the combination of fecal bacteria and viruses indicate a potential role in the diagnosis of difficult cases of CeD. In addition, identification of important bacteria as decreased abundances of *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in children with CeD, suggests a protective role with the potential for the development of preventive and adjuvant microbial therapy for CeD. The importance of *Human_endogenous _retrovirus_K* is interesting. However, further studies with larger sample sizes, are needed to improve our understanding of the role of the microbiota in CeD.

ARTICLE HIGHLIGHTS

Research background

Dysbiosis associated with celiac disease (CeD) is well known and beneficial and harmful associations have been reported.

Research motivation

The role of the microbiota in predicting CeD has rarely been described.

Research objectives

To search for a microbial signature that may help in the diagnosis and prevention of CeD.

Research methods

Metagenomic analysis of microbial DNA in mucosa and stool of children with newly diagnosed CeD calculation of the area under the curve to evaluate the predictive power of the whole microbiota and use of rendom forest analysis to identify important microbes in distinguishing CeD groups from controls.

Research results

Very high discriminatory power of combined bacteria and viruses (81.8%) in fecal samples and bacteria only in mucosal samples (81.2%). *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in fecal samples were demmed important.

Research conclusions

The excellent predictive power of microbiota may help in the diagnosis of difficult cases of CeD. The identification of important specific bacterial species that are reduced in CeD may have a potential protective role.

Research perspectives

Future research in this area with larger sample sizes is needed to clarify the role of microbiota in the diagnosis and prevention of CeD.

FOOTNOTES

Author contributions: El Mouzan M designed the study, interpreted the data, drafted, and revised the manuscript; Assiri A and Al Sarkhy A participated equally in the data management and revision; and all authors approved the final manuscript.

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Informed consent statement: All parents and children received informed consent/assent before participation in the study.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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ORIGINAL ARTICLE

Retrospective Study

Preoperative prediction of macrotrabecular-massive hepatocellular carcinoma through dynamic contrast-enhanced magnetic resonance imaging-based radiomics

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Abstract

BACKGROUND

Macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) is closely related to aggressive phenotype, gene mutation, carcinogenic pathway, and immunohistochemical markers and is a strong independent predictor of early recurrence and poor prognosis. With the development of imaging technology, successful applications of contrast-enhanced magnetic resonance imaging (MRI) have been reported in identifying the MTM-HCC subtype. Radiomics, as an objective and beneficial method for tumour evaluation, is used to convert medical images into high-throughput quantification features that greatly push the development of precision medicine.

AIM

To establish and verify a nomogram for preoperatively identifying MTM-HCC by comparing different machine learning algorithms.

METHODS

This retrospective study enrolled 232 (training set, 162; test set, 70) hepatocellular carcinoma patients from April 2018 to September 2021. A total of 3111 radiomics features were extracted from dynamic contrast-enhanced MRI, followed by dimension reduction of these features. Logistic regression (LR), K-nearest neighbour (KNN), Bayes, Tree, and support vector machine (SVM) algorithms

were used to select the best radiomics signature. We used the relative standard deviation (RSD) and bootstrap methods to quantify the stability of these five algorithms. The algorithm with the lowest RSD represented the best stability, and it was used to construct the best radiomics model. Multivariable logistic analysis was used to select the useful clinical and radiological features, and different predictive models were established. Finally, the predictive performances of the different models were assessed by evaluating the area under the curve (AUC).

RESULTS

The RSD values based on LR, KNN, Bayes, Tree, and SVM were 3.8%, 8.6%, 4.3%, 17.7%, and 17.4%, respectively. Therefore, the LR machine learning algorithm was selected to construct the best radiomics signature, which performed well with AUCs of 0.766 and 0.739 in the training and test sets, respectively. In the multivariable analysis, age [odds ratio (OR) = 0.956, P = 0.034], alpha-fetoprotein (OR = 10.066, P < 0.001), tumour size (OR = 3.316, P = 0.002), tumour-to-liver apparent diffusion coefficient (ADC) ratio (OR = 0.156, P = 0.037), and radiomics score (OR = 2.923, P < 0.001) were independent predictors of MTM-HCC. Among the different models, the predictive performances of the clinical-radiomics model and radiological-radiomics model were significantly improved compared to those of the clinical model (AUCs: 0.888 *vs* 0.836, P = 0.046) and radiological model (AUCs: 0.796 *vs* 0.688, P = 0.012), respectively, in the training set, highlighting the improved predictive performance of radiomics. The nomogram performed best, with AUCs of 0.896 and 0.805 in the training and test sets, respectively.

CONCLUSION

The nomogram containing radiomics, age, alpha-fetoprotein, tumour size, and tumour-to-liver ADC ratio revealed excellent predictive ability in preoperatively identifying the MTM-HCC subtype.

Key Words: Hepatocellular carcinoma; Macrotrabecular-massive subtype; Algorithms; Radiomics; Models; Nomogram

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Core Tip: Radiomics features can be used to predict the macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) subtype. The logistic regression algorithm can improve the accuracy and stability of predicting MTM-HCC. Age, alpha-fetoprotein, tumour size, tumour-to-liver apparent diffusion coefficient ratio, and radiomics score were significant independent predictors of MTM-HCC. The nomogram based on radiomics, clinical and radiological features can serve as a noninvasive biomarker to preoperatively identify MTM-HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide, with a 5-year recurrence rate of 70% after surgical resection[1]. The poor prognosis of patients with HCC is closely related to histopathological subtypes[2]. Recently, a newly identified histopathological subtype was named "macrotrabecular-massive HCC (MTM-HCC)" and was officially included in the new classification of HCC by the World Health Organization in 2019[3]. As the most common subtype with metastatic potential[4], MTM-HCC is closely related to gene mutation, carcinogenic pathway, and immunohistochemical markers[5] and is a strong independent predictor of early recurrence and poor prognosis[6,7].

Early diagnosis and appropriate treatment of MTM-HCC are beneficial to prevent early recurrence and improve prognosis. Current studies have shown that radiofrequency ablation is not recommended for patients with aggressive HCC, while performing resection with wide margins or anatomical hepatectomy and shorter follow-up intervals may be recommended for monitoring[8,9]. MTM-HCC shows an aggressive phenotype[10]. Therefore, an accurate preoperative diagnosis of MTM-HCC can

provide the best individualized treatment plan.

With the development of imaging technology, successful applications of magnetic resonance imaging (MRI) have been reported in identifying the MTM-HCC subtype[11]. Several studies[11-14] have reported that MTM-HCC has characteristic MRI features, such as intratumor substantial necrosis and intratumor fat. However, these studies mainly focused on the qualitative analysis of imaging features. Radiomics, as an objective and beneficial method for tumour evaluation, is used to convert medical images into high-throughput quantification features that greatly push the development of precision medicine[15]. A recent study has also shown that radiomics is a superior tool for predicting MTM-HCC. However, this study only enrolled 88 patients and lacked a validation set, and there was limited reproducibility of the results. In addition, a highly accurate and reliable radiomics model can be constructed by comparing different machine learning algorithms. Therefore, we hypothesize that valuable MRI-based radiomics features could be extracted and a noninvasive and comprehensive model could be constructed through machine learning to better predict MTM-HCC.

In this study, we aimed to apply MRI-based radiomics to preoperatively predict MTM-HCC using different machine learning algorithms to build the best radiomics signature and to establish and validate the nomogram by combining preoperative clinical and radiological features to improve the decision-making process in clinical practice.

MATERIALS AND METHODS

Patients

This retrospective study was approved by our institutional review board, and the requirement for written informed consent was waived. Between April 2018 and September 2021, patients with suspected HCCs who underwent preoperative liver MRI examinations were included. The inclusion criteria included the following: (1) Pathologically proven primary HCC; (2) received liver MRI examinations one month before surgery; and (3) underwent curative hepatectomy. The exclusion criteria included: (1) Tumours less than 1 cm; (2) preoperative antitumour treatments; (3) poor image quality caused by metal or motion artifacts; and (4) lack of complete clinicopathological data. Finally, 232 patients were enrolled and divided into training (n = 162) and test (n = 70) sets at a ratio of 7:3. The patient recruitment process is shown in Figure 1.

MRI examinations

All MRI examinations were performed on a 3.0 T MRI scanner (Discovery MR 750, GE Healthcare, Waukesha, WI, United States). Our liver MRI protocol included the following sequence: (1) Axial T_2-weighted imaging with fat suppression: Repetition time (TR)/echo time (TE), 13000/75 msec; field of view (FOV), 360 mm × 360 mm; matrix, 320 × 320; slice thickness, 5 mm; (2) dual-echo (in-phase and opposed-phase) T_1-weighted imaging: TR/TE, 3.7/1.7 msec; FOV, 360 mm × 288 mm; matrix, 260 × 224; slice thickness, 5 mm; (3) diffusion-weighted imaging (DWI) and apparent diffusion coefficient (ADC): TR/TE, 8000/50 msec; FOV, 360 mm × 288 mm; matrix, 128×96; and slice thickness, 5 mm. DWI was obtained using respiratory triggering, a single-shot echo-planar imaging pulse sequence with b values of 0 and 800 s/mm^2; and (4) The dynamic contrast-enhanced (DCE) three-dimensional fast-spoiled gradient-recalled echo sequences were as follows: TR/TE, 3.7/1.7 msec; FOV, 360 mm × 288 mm; matrix, 260 × 224; and slice thickness, 5 mm. The DCE sequences were acquired at 15-20 s (arterial phase, AP), 50-55 s (portal phase, PP) and 85-90 s (delayed phase, DP) after contrast-agent injection.

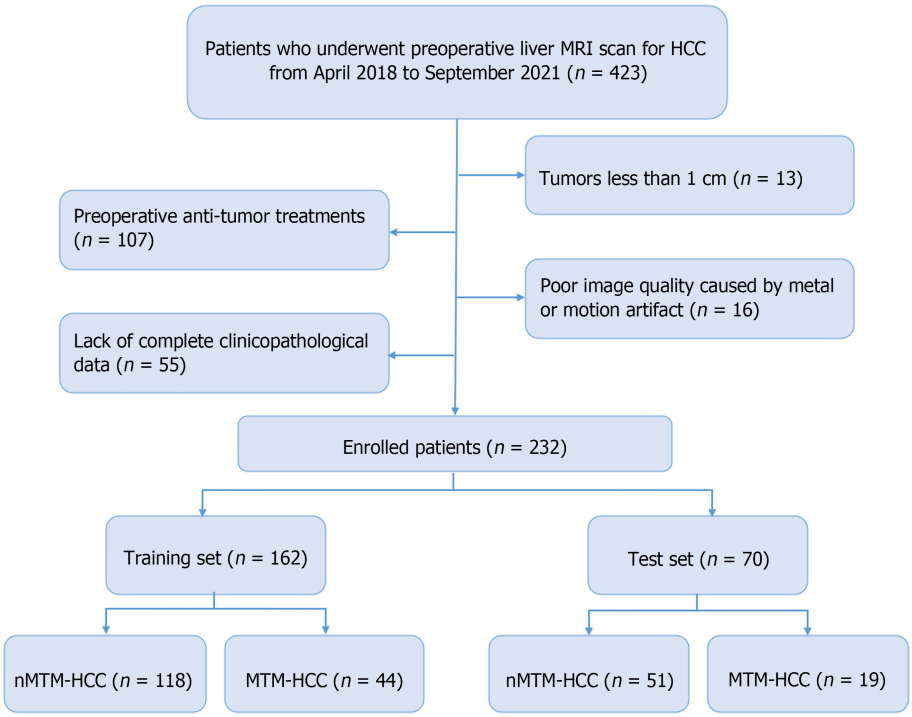
Clinical and radiological data

Clinical data included age, sex, hepatitis B surface antigen (HBsAg) status (positive or negative), serum alpha-fetoprotein (AFP) level (recorded as > 400 μg/L or ≤ 400 μg/L), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), platelet count (PLT), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), albumin, aspartate transaminase (AST), alanine transferase (ALT) and AST/ALT.

Radiological data of all patients were retrospectively analysed by two radiologists who were unaware of the clinicopathological findings and included liver cirrhosis, tumour size, tumour shape, intratumor fat, intratumor necrosis, intratumor haemorrhage, enhancing capsule and tumour-to-liver ADC ratio. For qualitative data, an agreement was reached by negotiation when there was disagreement between the two radiologists. For quantitative data, the measurements from these two radiologists were averaged. Detailed descriptions of the radiological features are shown in Supplementary Table 1.

Pathological data

All enrolled patients underwent hepatectomy, and pathological evaluations were performed in consensus by two experienced pathologists. MTM-HCC was defined as a predominant macrotrabecular architectural pattern (> 6 cells thick) involving more than 50% of the entire tumour[7]. nMTM-HCC was defined as non-MTM-HCC. The following pathological features were recorded: tumour differentiation



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Figure 1 The patient recruitment process. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging.

according to the Edmondson-Steiner grade (I-II or III-IV), presence of microvascular invasion, satellite nodules, and biliary invasion.

Radiomics features

Image segmentation: Image segmentation was performed with an open-source software named ITK-SNAP. The volume of interest (VOI) was defined by manually outlining the whole tumour border in AP, PP, and DP sequences slice-by-slice for each patient by a radiologist with five years of experience in liver MRI. Then, the segmentation results were validated by another radiologist with more than 10 years of experience in liver MRI using the intraclass correlation coefficient (ICC) on a cohort of 30 randomly selected patients. The image segmentation process is shown in Supplementary Figure 1.

Radiomics feature extraction: All segmented VOIs were loaded into the Pyradiomics-based PHIgo software (GE Healthcare, V1.2.0, China) for feature extraction, which complies with the image biomarker standardization initiative (IBSI)[16]. Before that, all images were subjected to standardized preprocessing, including resampling the images at the same resolution (1 mm × 1 mm × 1 mm) and classifying the grayscale into 1-10 Levels. Then, 1037 radiomics features were extracted from each VOI of each sequence, including the first-order features (first order: 18 features), shape-based features (shape: 14 features), gray-level run length matrix features (GLRLM: 16 features), gray-level size zone matrix (GLSZM: 16 features), neighbourhood gray tone difference matrix (NGTDM: 5 features), gray-level co-occurrence matrix features (GLCM: 24 features), gray-level dependence matrix (GLDM: 14 features), LoG transform features (LoG: 186 features), and wavelet transform features (wavelet: 744 features). The DCE sequences, including AP, PP, and DP, were scanned, affording 3111 radiomics features per patient.

Radiomics feature selection: The ICCs of the measurements from the two radiologists were applied to evaluate the interobserver reliability and reproducibility. The features with ICCs > 0.80 were considered robust features. Then, dimension reduction was performed using analysis of variance, correlation analysis, and gradient boosting decision tree (GBDT) to reduce data redundancy and to further select the most significant radiomics features based on DCE sequences.

Radiomics signature development: Five machine learning algorithms, including logistic regression (LR), K-nearest neighbour (KNN), Bayes, Tree, and support vector machine (SVM), were used to construct radiomics signatures based on the retained significant features. Then, we used the relative standard deviation (RSD) and bootstrap methods to quantify the stability of these five algorithms. The algorithm with the lowest RSD represented the best stability, which was used to construct the best radiomics model. Finally, the radiomics score (rad-score) was calculated *via* a linear combination of the

remaining features that were weighted by their respective coefficients to quantify the discriminability of the radiomics model. Details of the RSD can be found in the Supplementary materials.

Model construction and evaluation

Univariate logistic analysis was performed on variables, including the abovementioned clinical features, radiological features, and rad-score. Variables with $P < 0.05$ were included in the multivariate logistic analysis to determine the potential independent predictors of MTM-HCC, based on which combined model was built. In addition, the clinical model was constructed based on the final selected clinical features, and the radiological model was constructed based on the final selected radiological features. To verify the improvement in the performance of the model after including radiomics, we integrated the selected independent predictors to construct different fusion models. The area under the receiver operating characteristic (ROC) curve (AUC) and the DeLong test were used to evaluate the performance of the different models for predicting MTM-HCC. The Hosmer–Lemeshow test was used to assess the goodness-of-fit of the combined model. A nomogram based on the combined model was established for easy use to generate a probability of MTM-HCC. Then, the patients were classified into high-risk and low-risk groups according to the nomogram. The flowchart of the model construction and evaluation is shown in Figure 2.

Statistical analysis

Statistical analyses were performed with SPSS software (version 24.0, Chicago, IL, United States) and R software (version 3.4.1, Vienna, Austria). Continuous variables were expressed as the mean ± SD or median (interquartile range). Categorical variables are presented as numbers (percentages). Continuous variables were analysed using a two-sample *t* test or Mann-Whitney *U* test if not normally distributed. The chi-squared test or Fisher's exact test was used for categorical variables. Statistical significance was set at a two-sided $P < 0.05$.

RESULTS

Patient characteristics

Clinical, pathological, and radiological features of the HCC patients are shown in Tables 1 and 2. In terms of the MTM-HCC and nMTM-HCC groups, age, PLT, tumour size, intratumor haemorrhage, and tumour-to-liver ADC ratio were significantly different in both the training and test sets ($P < 0.05$). In addition, AFP > 400 μg/L ($P < 0.001$), CA19-9 ($P = 0.021$), AST ($P = 0.004$), ALT ($P = 0.023$), tumour shape ($P = 0.024$), and intratumor necrosis ($P = 0.005$) also differed significantly between the two groups in the training set. The Edmondson-Steiner grade ($P = 0.048$) differed significantly between the two groups in the test set. There were no significant differences in any of the features between the training and test sets ($P > 0.05$).

Radiomics signature development

A total of 3111 radiomics features were extracted for each patient based on the DCE sequences. Then, 2417 features with ICCs > 0.80 were obtained as robust features. After feature selection by analysis of variance and correlation analysis, 75 features were selected. Following GBDT, 27 features were ultimately retained, as shown in Figure 3A. The RSD values based on LR, KNN, Bayes, Tree, and SVM were 3.8%, 8.6%, 4.3%, 17.7%, and 17.4%, respectively. Therefore, the LR machine learning algorithm was chosen to construct the best radiomics signature (Figure 3B). The corresponding rad-score was calculated and was significantly different between the MTM-HCC and nMTM-HCC groups in the training set ($P < 0.001$) and test set ($P = 0.002$), as shown in Figure 3C. Details of the retained radiomics features and rad-score are shown in Supplementary Table 2 and Supplementary Figure 2.

Model construction and comparison

The multivariate logistic analysis showed that age (OR = 0.956, $P = 0.034$), AFP (OR = 10.066, $P < 0.001$), tumour size (OR = 3.316, $P = 0.002$), tumour-to-liver ADC ratio (OR=0.156, $P = 0.037$), and rad-score (OR = 2.923, $P < 0.001$) were independent predictors of MTM-HCC (Table 3). A clinical model was constructed based on age and AFP. A radiological model was constructed based on tumour size and the tumour-to-liver ADC ratio. To verify the improvement in the performance of the model after including radiomics, we constructed different fusion models, including a clinical-radiomics model and a radiological-radiomics model. Finally, we integrated all five of the selected independent predictors to build the combined model. The corresponding model score of the combined model was calculated, as shown in Supplementary Figure 3.

Among the six different models, the combined model performed best, with AUCs of 0.896 and 0.805 in the training and test sets, respectively (Table 4). The DeLong test showed that the predictive performances of the clinical-radiomics model and radiological-radiomics model were significantly improved compared to those of the clinical model (AUCs: 0.888 *vs* 0.836, $P = 0.046$) and radiological

Table 1 Clinical and pathological features in the training and test sets

Characteristics	Training set (*n* = 162)			Test set (*n* = 70)			*P* value
	nMTM-HCC (*n* = 118)	MTM-HCC (*n* = 44)	*P* value	nMTM-HCC (*n* = 51)	MTM-HCC (*n* = 19)	*P* value	
Age, mean ± SD	60.6 ± 10.9	52.4 ± 9.9	< 0.001	60.7 ± 10.4	54.7 ± 9.7	0.034	0.647
Sex (men, %)	102 (86.4)	42 (95.5)	0.179	39 (76.5)	18 (94.7)	0.161	0.125
HBsAg (positive, %)	91 (77.1)	33 (75.0)	0.777	38 (74.5)	14 (73.7)	0.944	0.712
AFP > 400 μg/L, *n* (%)	18 (15.3)	31 (70.5)	< 0.001	12 (23.5)	9 (47.4)	0.053	0.970
CEA (ug/L)	2.6 (2.1)	2.7 (1.9)	0.719	2.2 (2.0)	2.2 (1.2)	0.543	0.332
CA19-9 (U/mL)	15.0 (19.3)	28.0 (25.4)	0.021	16.7 (17.1)	16.0 (34.4)	0.687	0.770
PLT (× 10^9/L)	135.0 (83.0)	174.0 (88.5)	0.027	135.0 (57.0)	175.0 (127.0)	0.001	0.970
TBIL (μmol/L)	16.6 (10.8)	20.7 (9.8)	0.067	15.7 (10.3)	14.4 (7.6)	0.916	0.509
DBIL (μmol/L)	3.8 (2.6)	4.8 (2.9)	0.060	3.2 (2.1)	3.3 (2.7)	0.620	0.419
IBIL (μmol/L)	12.5 (7.0)	15.0 (7.4)	0.136	12.2 (7.8)	10.9 (4.5)	0.712	0.061
Albumin, mean ± SD	37.8 ± 5.0	37.0 ± 5.8	0.387	37.8 ± 5.0	38.3 ± 5.1	0.715	0.647
AST (U/L)	32.0 (23.5)	43.0 (68.8)	0.004	33.0 (29.0)	43.0 (61.0)	0.135	0.980
ALT (U/L)	31.5 (34.0)	46.0 (64.0)	0.023	28.0 (35.0)	57.0 (96.0)	0.074	0.748
AST/ALT	1.1 (0.6)	1.1 (0.5)	0.702	1.3 (0.5)	1.1 (0.7)	0.207	0.842
Edmondson-Steiner grade (III-IV, %)	38 (32.2)	18 (40.9)	0.300	14 (27.5)	10 (52.6)	0.048	0.967
Microvascular invasion, *n* (%)	56 (47.5)	20 (45.5)	0.820	18 (35.3)	10 (52.6)	0.188	0.331
Satellite nodules, *n* (%)	9 (7.6)	5 (11.4)	0.452	1 (2.0)	1 (5.3)	0.461	0.110
Biliary invasion, *n* (%)	4 (3.4)	1 (2.3)	0.715	2 (3.9)	1 (5.3)	0.805	0.646

Data in parentheses are presented as median (interquartile range) or *n* (%). MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; PLT: Platelet count; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; AST: Aspartate transaminase; ALT: Alanine transferase; SD: Standard deviation.

model (AUCs: 0.796 *vs* 0.688, $P = 0.012$), respectively, in the training set, highlighting the improved predictive performance of radiomics. In addition, the combined model performed better than the radiological model ($P < 0.001$), radiomics model ($P = 0.001$), and radiological-radiomics model ($P = 0.002$) in the training set, and the combined model was significantly different from the clinical model in both the training set ($P = 0.023$) and the test set ($P = 0.042$) (Table 4 and Figure 4).

Nomogram building and verification

Based on the combined model, we developed an intuitive, simple-to-use nomogram for individual risk prediction of MTM-HCC (Figure 5). The Hosmer–Lemeshow test exhibited good calibration of the nomogram in the training set ($P = 0.995$) and test set ($P = 0.466$). According to the optimal cut-off value of -1.663, the patients were stratified into low-risk and high-risk groups. As shown in Figure 6, there were significant differences in the number of patients who were predicted to have MTM-HCC between the low-risk and high-risk groups in the training set ($P < 0.001$), test set ($P = 0.003$), and all study populations ($P < 0.001$), indicating the clinical applicability of the nomogram.

DISCUSSION

In this study, we comprehensively evaluated clinical and radiological features and found that age, AFP, tumour size, and tumour-to-liver ADC ratio were significant independent predictors of MTM-HCC. By comparing five different machine learning algorithms (LR, KNN, Bayes, Tree, and SVM), we finally selected the LR algorithm with the best stability to construct the radiomics signature. Finally, by further comparing the predictive performance of the different models, the optimal combined model was

Table 2 Radiological features of patients in the training and test sets

Radiological features	Training set (*n* = 162)			Test set (*n* = 70)			*P* value
	nMTM-HCC (*n* = 118)	MTM-HCC (n = 44)	*P* value	nMTM-HCC (*n* = 51)	MTM-HCC (*n* = 19)	*P* value	
Liver cirrhosis (positive, %)	69 (58.5)	29 (65.9)	0.389	34 (66.7)	15 (78.9)	0.319	0.168
Tumour size > 5 cm, *n* (%)	29 (24.6)	22 (50.0)	0.002	12 (23.5)	10 (52.6)	0.020	0.994
Tumour shape (irregular, %)	25 (21.2)	17 (38.6)	0.024	17 (33.3)	7 (36.8)	0.783	0.195
Intratumor fat, *n* (%)	24 (20.3)	10 (22.7)	0.740	11 (21.6)	1 (5.3)	0.210	0.500
Intratumor necrosis, *n* (%)	34 (28.8)	23 (52.3)	0.005	13 (25.5)	8 (42.1)	0.177	0.443
Intratumor hemorrhage, *n* (%)	24 (20.3)	17 (38.6)	0.017	7 (13.7)	8 (42.1)	0.025	0.526
Enhancing capsule, *n* (%)	85 (72.0)	35 (79.5)	0.332	39 (76.5)	15 (78.9)	0.826	0.620
Tumour-to-liver ADC ratio	0.9 (0.2)	0.8 (0.3)	0.018	0.9 (0.1)	0.8 (0.2)	0.045	0.312

Data in parentheses are presented as median (interquartile range) or *n* (%). MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; ADC: Apparent diffusion coefficient.

Table 3 Results of univariate and multivariate logistic regression analyses

Variables	Univariate logistic regression		Multivariate logistic regression	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Clinical features				
Age	0.930 (0.896, 0.964)	< 0.001	0.956 (0.918, 0.997)	0.034
AFP > 400 μg/L	13.248 (5.839, 30.058)	< 0.001	10.066 (4.304, 23.541)	< 0.001
PLT	1.006 (1.002, 1.011)	0.009	NA	NA
Radiological features				
Tumour size > 5 cm	3.069 (1.487, 6.333)	0.002	3.316 (1.579, 6.962)	0.002
Tumour shape	2.342 (1.106, 4.961)	0.026	NA	NA
Intratumor necrosis	2.706 (1.326, 5.521)	0.006	NA	NA
Intratumor hemorrhage	2.466 (1.160, 5.244)	0.019	NA	NA
Tumour-to-liver ADC ratio	0.183 (0.035, 0.972)	0.046	0.156 (0.027, 0.894)	0.037
Radiomics				
Rad-score	2.718 (1.809, 4.084)	< 0.001	2.923 (1.740, 4.911)	< 0.001

AFP: Alpha-fetoprotein; PLT: Platelet count; ADC: Apparent diffusion coefficient; OR: Odds ratio; CI: Confidence interval; NA: Not available.

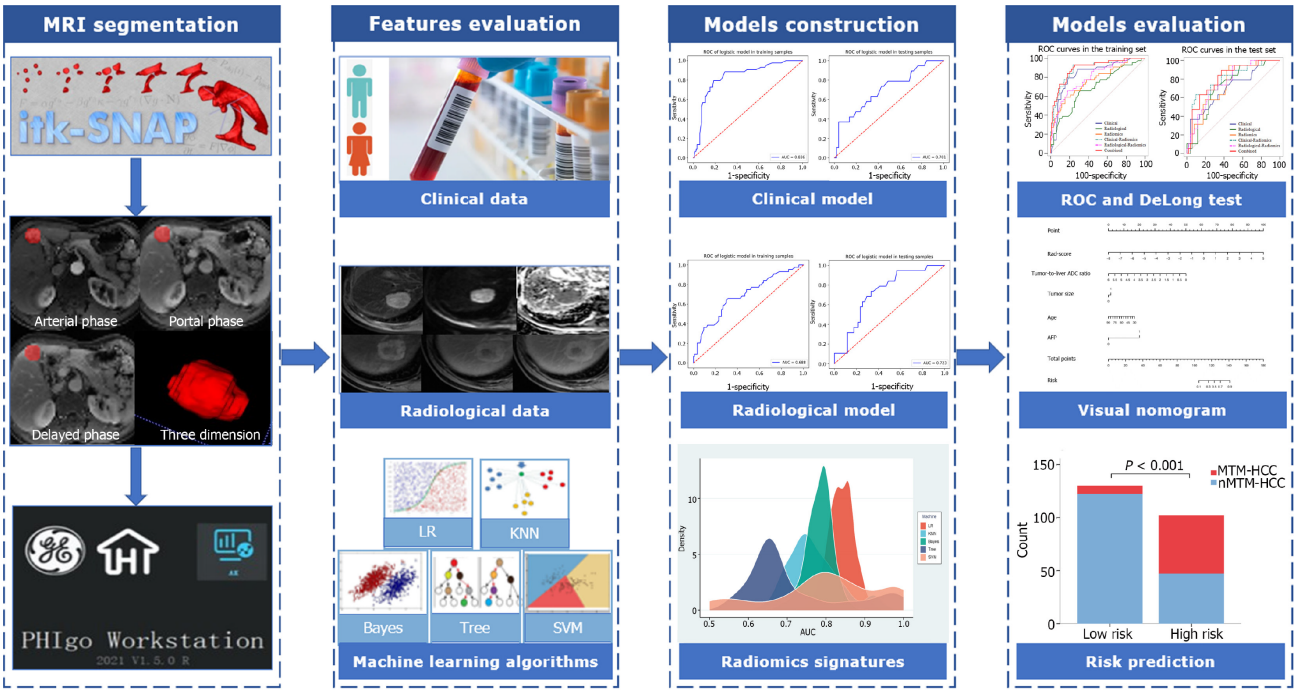
selected, and a visual nomogram was constructed, which provided a reliable theoretical basis for the development of a simple, easy-to-use, and accurate assessment tool and indicated that it had great potential in the field of preoperative noninvasive prediction of MTM-HCC.

Although some previous studies have focused on the clinical and radiological features of MTM-HCC, there is no consensus on the best biomarker for predicting MTM-HCC. Our study showed that MTM-HCC patients presented with large tumour sizes and high AFP loads, which were consistent with previous findings[17-20]. Interestingly, this study found that the age of patients with MTM-HCC was lower than that of the patients with nMTM-HCC. This may be because the activation of angiogenesis is the reason for the unique invasive biological characteristics of such tumours. Overexpression of angiopoietin 2 and vascular endothelial growth Factor A can cooperatively promote new angiogenesis in MTM-HCC, and these factors are indicators of poor prognosis in solid tumours[6,21]. This also confirmed that the aggressiveness of MTM-HCC may be related to molecular factors. Various studies

Table 4 Predictive performance of different models in training and test sets

Models	Training set				Test set			
	AUC (95%CI)	Accuracy	Sensitivity	Specificity	AUC (95%CI)	Accuracy	Sensitivity	Specificity
Clinical	0.836 (0.773-0.888)	0.802	0.750	0.822	0.701 (0.575-0.814)	0.671	0.579	0.706
Radiological	0.688 (0.604-0.769)	0.685	0.636	0.703	0.723 (0.610-0.829)	0.700	0.632	0.725
Radiomics	0.766 (0.692-0.836)	0.772	0.568	0.847	0.739 (0.634-0.837)	0.743	0.579	0.804
Clinical-radiomics	0.888 (0.835-0.934)	0.802	0.909	0.763	0.793 (0.682-0.893)	0.686	0.737	0.667
Radiological-radiomics	0.796 (0.725-0.858)	0.772	0.636	0.822	0.764 (0.661-0.859)	0.729	0.632	0.765
Combined	0.896 (0.847-0.939)	0.796	0.932	0.746	0.805 (0.704, 0.895)	0.700	0.895	0.628

AUC: Area under the receiver operating characteristic curve; CI: Confidence interval.

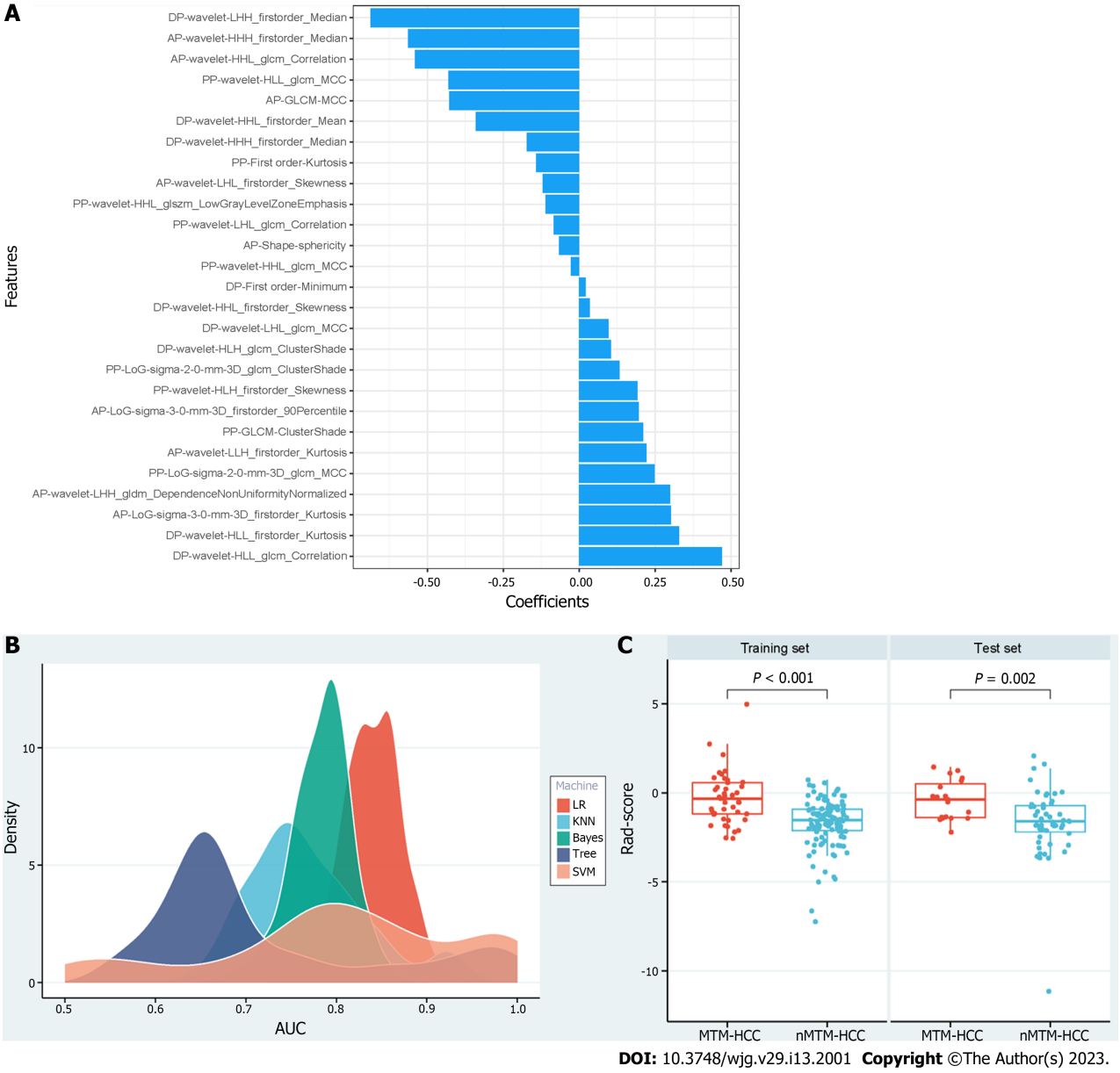


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Figure 2 The radiomics workflow. LR: Logistic regression; KNN: K-nearest neighbour; SVM: Support vector machine; ROC: Receiver operating characteristic; MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

have explored the relationship between PLT and MTM-HCC, but no consensus has been reached[22]. Our study showed that PLT was not an independent predictor. The multivariate logistic analysis showed that a low tumour-to-liver ADC ratio was an independent predictor of MTM-HCC, which was consistent with the results of Chen *et al*[12]. This is because the cellular structure is increased, the arterial supply is reduced, and diffusion is more restricted in more aggressive HCC[5]. In addition, previous studies[11,20] have suggested that intratumor necrosis can be used as an independent predictor of MTM-HCC. However, in our study, although the univariate logistic analysis showed that intratumor necrosis was more common and statistically significant in MTM-HCC, it was not an independent predictor, which was also consistent with the results of Zhu *et al*[14]. This may be because intratumor necrosis is not a commonly reported finding of aggressiveness or poor prognosis in HCC and lacks high specificity. This finding was more common in non-HCC malignancies, such as intrahepatic cholangiocarcinoma or metastasis, and therefore was considered a feature of non-HCC malignancies[23].

At present, there is no research on the value of different machine learning algorithms in predicting MTM-HCC. In this study, we compared the performance of radiomics signatures constructed by five machine learning algorithms, and the results showed that the radiomics signature constructed by the LR algorithm had the best stability, with an RSD of 3.8%. The radiomics model based on the LR algorithm performed well in predicting MTM-HCC, with AUCs of 0.766 and 0.739 and specificities of 0.847 and 0.804 in the training and test sets, respectively. Therefore, radiomics is very useful for timely capturing

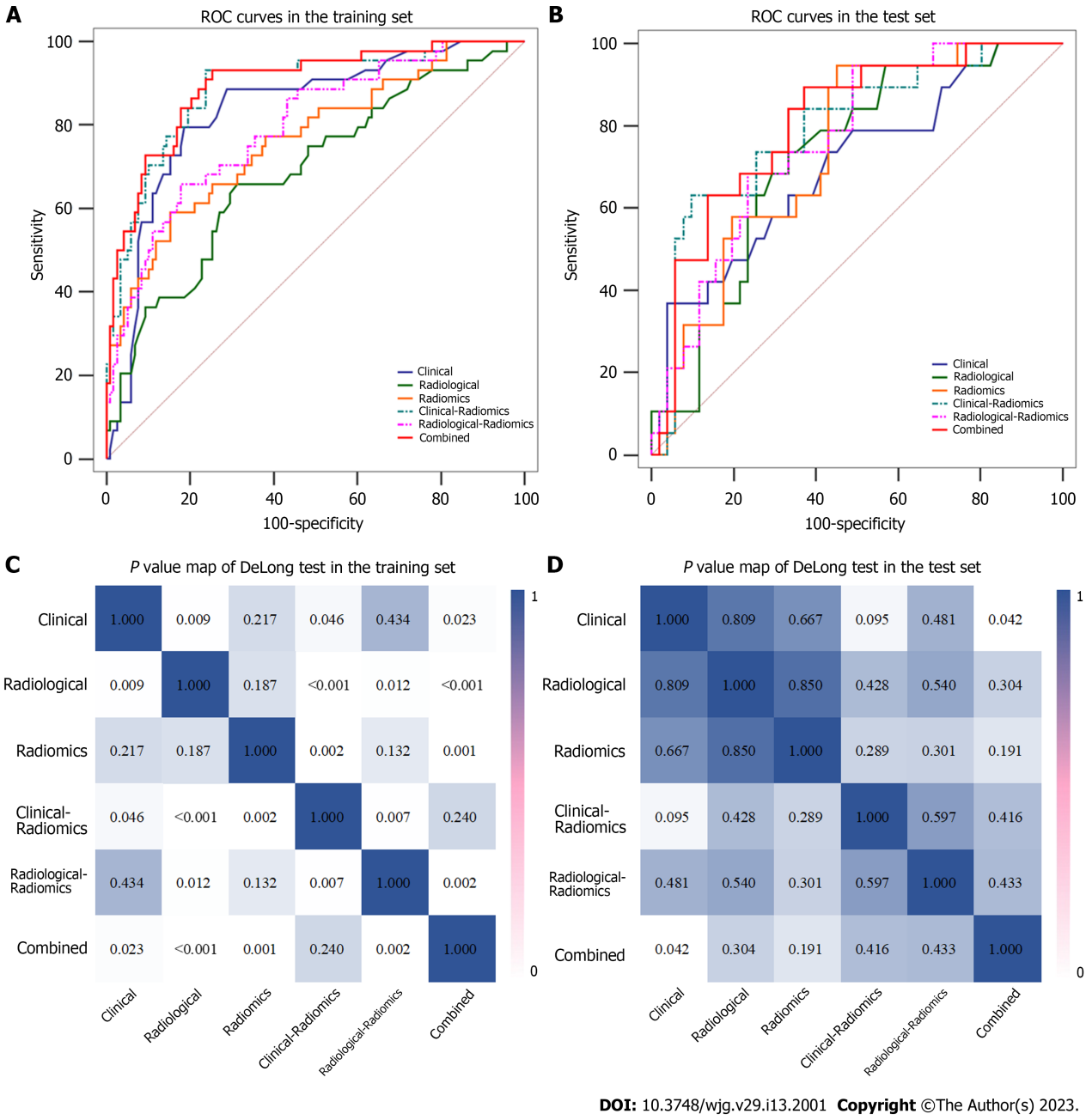


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Figure 3 Radiomics signature development. A: Of 27 selected radiomics features and their coefficients; B: Density distribution of the area under the receiver operating characteristic curve of the radiomics signatures constructed by the five machine learning algorithms; C: Box-scatter plots show that rad-score of macrotrabecular-massive hepatocellular carcinoma is substantially higher than that of non-macrotrabecular-massive hepatocellular carcinoma in both the training and test sets. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

and reflecting the underlying histopathological features. In addition, 27 features were finally retained in this study, of which 22 (81.5%) features (4 LoG and 18 wavelet features) were extracted from the original and derived images[24]. For example, this study confirmed that "cluster shade", "correlation" and "MCC" were the most meaningful features among GLCM, showing differences in the regional signal intensity distribution and linear dependency. Among the first-order features, “Mean” and “Median” describe the mean and median grey intensity of the voxel intensity within the tumour region, respectively, and the differences in the grey intensity distribution are represented by “Kurtosis” and “Skewness”. “GISZM_LowGrayLevelZoneEmphasis” and “GLDM_DependenceNonUniformityNormalized” represent the heterogeneity of the tumour. These results are consistent with some radiomics features extracted from previous studies on the pathological and survival prediction of HCC[25-27], indicating that LoG and wavelet features can represent signal intensity distribution or grey distribution in tissues, which can better reflect the biological characteristics and heterogeneity of tumours.

Our study found that the clinical model based on age and AFP (AUC, 0.836; sensitivity, 0.750; specificity, 0.822) had a significantly higher diagnostic efficiency than the clinical model based on AST, PT, and AFP proposed by Shan *et al*[28] (AUC, 0.723; sensitivity, 0.711; specificity, 0.607) in predicting MTM-HCC. The limitation of our clinical model was that it had poor performance in the test set, with an AUC of 0.701, a sensitivity of 0.579, and a specificity of 0.706. However, when the radiomics signature was introduced, the predictive performances of the clinical-radiomics model and radiological-radiomics

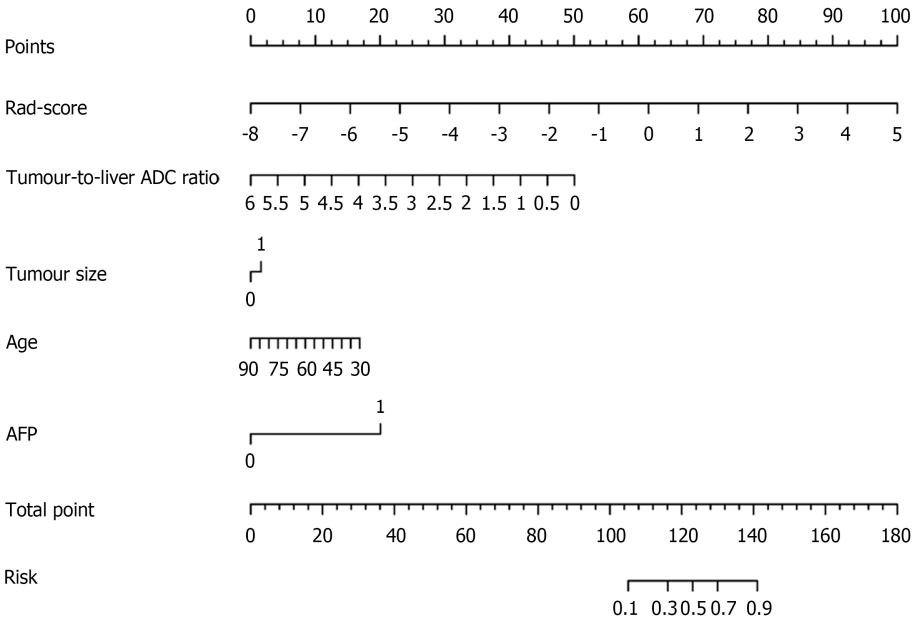


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Figure 4 Predictive performance of different models. A and B: Receiver operating characteristic curves of different models in the training and test sets; C and D: *P* value map of DeLong test between the different models in the training and test sets. ROC: Receiver operating characteristic.

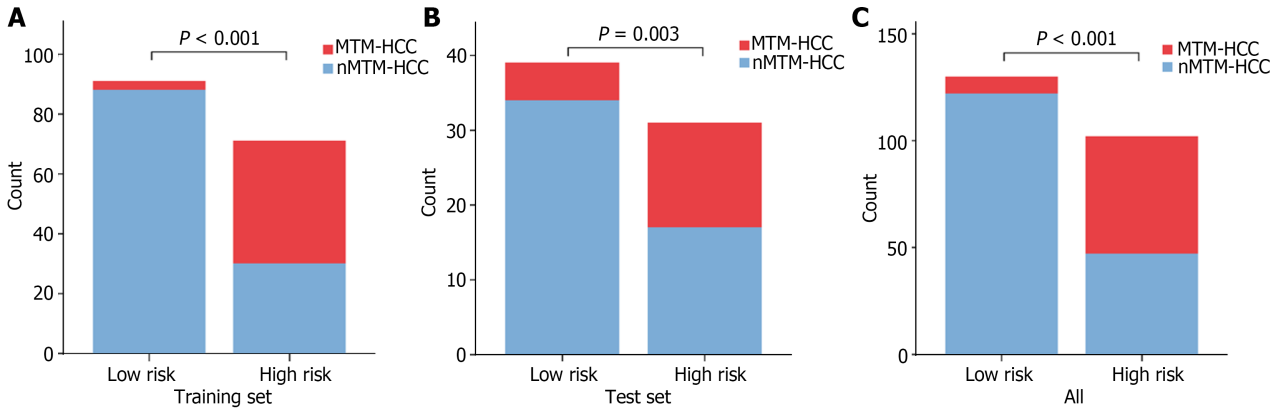
model were improved compared to the clinical model (AUCs: 0.888 *vs* 0.836 in the training set, 0.793 *vs* 0.701 in the test set) and radiological model (AUCs: 0.796 *vs* 0.688, 0.764 *vs* 0.723 in the test set), respectively, highlighting the improved predictive performance of radiomics. This is due to the unprecedented opportunities for extracting potential quantitative information from images provided by advanced radiomics analysis. Traditional imaging diagnosis usually relies on morphological changes observed by the naked eye. However, it often takes a long time for the appearance of observable morphological image changes of tumours caused by pathological changes. This also indicates that it is valuable for clinical applications to extract the quantitative radiomics features behind the images.

Since the diagnosis of HCC does not require biopsy, preoperative noninvasive identification of the MTM-HCC subtype is critical for treatment and prognosis. There was only one previous study on radiomics in the prediction of MTM-HCC, and the nomogram established based on radiomics and intratumor fat showed satisfactory prediction performance, with an AUC of 0.785[14]. However, that small sample study (n = 88) lacked a test set, and the reproducibility of the results was limited. It is worth mentioning that our nomogram performed best in the preoperative noninvasive prediction of MTM-HCC, with AUCs of 0.896 and 0.805 in the training and test sets, respectively. This study achieves the first step towards the noninvasive evaluation of MTM-HCC using radiomics and clinical and radiological features in clinical practice and may guide the selection of patients for whom targeted



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Figure 5 Visual nomogram construction. ADC: Apparent diffusion coefficient; AFP: Alpha-fetoprotein.



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Figure 6 Risk prediction based on the nomogram. Patients were divided into high-risk and low-risk groups according to the nomogram. The probability of pathological macrotrabecular-massive hepatocellular carcinoma in the high-risk group was significantly higher than that in the low-risk group in the training set, test set, and all study populations. A: Training set; B: Test set; C: All study populations. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

therapies would be effective. This again suggested the application value of radiomics in predicting MTM-HCC. Integrating multidimensional features is of great significance for building a powerful prediction model.

Some limitations should be noted. First, this retrospective study only included surgically resected lesions, which may cause selection bias. Therefore, the results of this study may not be representative of the entire clinical spectrum. Second, all patients were recruited from a single center, which may limit the external validation. Therefore, further studies using larger, multicenter samples are needed to verify our findings.

CONCLUSION

In this study, we found that age, AFP, tumour size, and tumour-to-liver ADC ratio were significant independent predictors of MTM-HCC. We compared and selected the optimal LR machine learning algorithm to construct the radiomics signature. The nomogram showed excellent predictive ability in preoperatively identifying MTM-HCC and showed great potential in clinical application, which was helpful to guide individualized treatment and improve the long-term survival outcomes of HCC

patients.

ARTICLE HIGHLIGHTS

Research background

Macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) shows an aggressive phenotype. Early diagnosis of MTM-HCC is beneficial to prevent early recurrence and improve prognosis. Radiomics can convert medical images into high-throughput quantification features, which greatly push the development of precision medicine.

Research motivation

Currently, magnetic resonance imaging (MRI) features have been successfully applied to identify MTM-HCC but have mainly focused on the qualitative analysis of imaging features. In this study, we systematically analysed radiomics, clinical and radiological features to build a more comprehensive prediction model. We aimed to develop a noninvasive model for the preoperative prediction of MTM-HCC.

Research objectives

In this study, we aimed to establish and verify a nomogram based on contrast-enhanced MRI for preoperatively identifying MTM-HCC by comparing different machine learning algorithms.

Research methods

A total of 232 (training set, 162; test set, 70) hepatocellular carcinoma patients were enrolled. Radiomics features were extracted from contrast-enhanced MRI, followed by dimension reduction. Logistic regression (LR), K-nearest neighbour, Bayes, Tree, and support vector machine algorithms were used to construct radiomics signatures. The relative standard deviation (RSD) was used to quantify the stability of these five algorithms. Multivariable logistic analysis was used to select the useful clinical and radiological features, and different predictive models were established. The performances of the different models were assessed using the area under the curve (AUC).

Research results

The LR algorithm with the smaller RSD (3.8%) was used to construct the best radiomics signature, which performed well with AUCs of 0.766 and 0.739 in the training and test sets, respectively. Age, alpha-fetoprotein, tumour size, tumour-to-liver apparent diffusion coefficient ratio, and radiomics score were identified as independent predictors of MTM-HCC to build the nomogram, which performed best with AUCs of 0.896 and 0.805 in the training and test sets, respectively.

Research conclusions

The nomogram is a reliable tool for preoperatively identifying the MTM-HCC subtype.

Research perspectives

More precise and reliable tools are urgently needed to predict the MTM-HCC subtype. Radiomics is a new method to convert medical images into high-throughput quantification features. In this study, we aimed to develop a dynamic contrast-enhanced MRI-based nomogram for preoperatively identifying the MTM-HCC subtype.

FOOTNOTES

Author contributions: Shi LL was the guarantor and designed the study; Zhang Y, He D, Liu J, and Shi LL participated in the acquisition, analysis, and interpretation of the data; Wei YG reviewed statistical methods; Zhang Y drafted the initial manuscript; Shi LL revised the article critically for important intellectual content; all authors have read and approve the final manuscript.

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Informed consent statement: Written informed consent was not required for this study because of retrospective study.

Conflict-of-interest statement: There are no conflicts of interest to report.

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ORIGINAL ARTICLE

Observational Study

Changes in characteristics of patients with hepatitis C virus-related cirrhosis from the beginning of the interferon-free era

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Abstract

BACKGROUND

Nearly 290000 patients with chronic hepatitis C die annually from the most severe complications of the disease. One of them is liver cirrhosis, which occurs in about 20% of patients chronically infected with the hepatitis C virus (HCV). Direct-acting antivirals (DAAs), which replaced interferon (IFN)-based regimens, significantly improved the prognosis of this group of patients, increasing HCV eradication rates and tolerability of therapy. Our study is the first to assess changes in patient profile, effectiveness, and safety in the HCV-infected cirrhotic population in the IFN-free era.

AIM

To document changes in patient characteristics and treatment regimens along with their effectiveness and safety profile over the years.

METHODS

The studied patients were selected from 14801 chronically HCV-infected individuals who started IFN-free therapy between July 2015 and December 2021 in 22 Polish hepatology centers. The retrospective analysis was conducted in real-world clinical practice based on the EpiTer-2 multicenter database. The measure of treatment effectiveness was the percentage of sustained virologic response (SVR) calculated after excluding patients lost to follow-up. Safety data collected during therapy and the 12-wk post-treatment period included information on adverse events, including serious ones, deaths, and treatment course.

RESULTS

The studied population (n = 3577) was balanced in terms of gender in 2015-2017, while the following years showed the dominance of men. The decline in the median age from 60 in 2015-2016 to 57 years in 2021 was accompanied by a decrease in the percentage of patients with comorbidities and comedications. Treatment-experienced patients dominated in 2015-2016, while treatment-naive individuals gained an advantage in 2017 and reached 93.2% in 2021. Genotype (GT)-specific options were more prevalent in treatment in 2015-2018 and were supplanted by pangenotypic combinations in subsequent years. The effectiveness of the therapy was comparable regardless of the period analyzed, and patients achieved an overall response rate of 95%, with an SVR range of 72.9%-100% for the different therapeutic regimens. Male gender, GT3 infection, and prior treatment failure were identified as independent negative predictors of therapeutic success.

CONCLUSION

We have documented changes in the profile of HCV-infected cirrhotic patients over the years of accessibility to changing DAA regimens, confirming the high effectiveness of IFN-free therapy in all analyzed periods.

Key Words: Hepatitis C; Liver cirrhosis; Direct-acting antivirals; Pangenotypic; Genotype-specific; Epidemiology

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Core Tip: Patients with cirrhosis in the course of chronic infection with the hepatitis C virus, in whom the risk of death due to advanced liver disease is the highest, seem to be the greatest beneficiaries of the introduction of therapies with direct-acting antiviral drugs. Our analysis tracking changes in the profile of these patients documents the very high effectiveness and good safety profile from the beginning of the interferon-free era to the present.

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INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) remains a global health problem, affecting 58 million people worldwide, according to the most recent estimates of the World Health Organization (WHO) published in 2022[1]. Despite its initially asymptomatic course, untreated chronic hepatitis C (CHC) can lead to progressive liver disease resulting in cirrhosis. Among HCV-infected patients, about 20% are at risk of developing cirrhosis after an average of about 20 years of infection[2], and 3%-6% of them develop decompensation of liver function each year[3]. Hepatic decompensation and hepatocellular carcinoma (HCC) are reported as the most severe complications of CHC, causing globally nearly 290000 deaths each year[1].

The antiviral treatment of patients with cirrhosis, as they are at risk of developing these most severe and potentially fatal complications, is crucial in achieving the WHO goal formulated in 2016, which assumes the reduction of mortality due to HCV by 65% by 2030[1]. In the era of interferon (IFN)-based antiviral regimens, therapeutic options were severely limited due to contraindications and poor safety profile in this patient population[4]. Even the introduction in 2011 of the first direct-acting antivirals (DAAs) and HCV protease inhibitors (telaprevir and boceprevir), which were registered for use with pegylated IFN, was not a breakthrough for this group of patients due to the limitations still associated with IFN[5]. Only the availability of IFN-free therapies based exclusively on a combination of DAA agents has significantly improved safety parameters and treatment efficacy, representing a real revolution in managing HCV-infected patients with cirrhosis, including those with decompensation[6]. As a result, at the beginning of the DAA era, cirrhotic patients were prioritized in access to treatment, with some countries even limiting therapy reimbursement to this group of patients[7]. The findings of a number of clinical trials and real-world experience (RWE) studies involving this particular patient population published to date indicate that, despite the lack of contraindications to DAA therapy, the presence of cirrhosis is still considered an unfavorable factor for achieving a sustained virologic response (SVR)[8,9].

Recently, several RWE studies have been conducted in different world parts, summarizing the effectiveness of DAA treatment and changes in demographic and clinical characteristics in HCV-infected patients treated with DAA regimens[10-14]. However, none of these have focused on cirrhotic patients, while filling key knowledge gaps regarding this population could support the WHO's goal of eliminating HCV as a major public health burden by 2030.

Therefore, the present study aimed to track changes in the profile of HCV-infected patients with cirrhosis treated with DAA options, along with documenting the evolution of antiviral regimens in RWE practice over seven years of access to IFN-free therapy. To this end, a retrospective analysis of the Polish population of HCV-infected patients with cirrhosis who were treated between 2015 and 2021 was conducted.

MATERIALS AND METHODS

Study population

The study population was selected from 14801 adult patients infected with HCV who started IFN-free antiviral treatment in 22 Polish hepatology centers from the beginning of the DAA availability between July 1, 2015 and December 31, 2021. The analysis was a part of the EpiTer-2 database, a retrospective national study evaluating antiviral therapy of HCV-infected patients in routine clinical practice, supported by the Polish Association of Epidemiologists and Infectiologists. The present study consisted of all consecutive CHC patients with liver cirrhosis treated with IFN-free therapy, reimbursed by the Polish National Health Fund (NFZ). Since the beginning of the availability of DAA regimens in Poland, there have been no restrictions related to the severity of liver disease or history of previous therapy in the qualification of patients for antiviral treatment. The choice of regimen, dose, and length of treatment course was at the discretion of the treating physician based on the available therapeutic options, and treatment was administered following the product characteristics, the protocol of the NFZ therapeutic program, and the recommendations of the Polish Group of Experts for HCV[15-19]. Before starting treatment, the patient signed a consent form as required by the current regulations of the therapeutic program.

Data collection

The data were collected retrospectively with an online questionnaire operated by Tiba LLC based on the medical records. The study was carried out by comparison of six groups of patients diagnosed with liver cirrhosis who were divided based on the time of treatment initiation: 2015-2016, 2017, 2018, 2019, 2020, and 2021. Parameters gathered at baseline included demographic and clinical data: Gender, age, body mass index, HCV genotype (GT), comorbidities, concomitant medications, information on the severity of the liver disease, coinfections of the human immunodeficiency virus (HIV) and hepatitis B virus (HBV), and history of previous antiviral therapy. Baseline laboratory parameters were recorded, including serum alanine transaminase activity, bilirubin concentrations, albumin, creatinine, hemoglobin, platelet count, and HCV viral load. The patient groups were compared regarding the treatment regimens used and their efficacy and safety outcomes.

Assessment of liver disease severity

The advancement of liver disease was evaluated by assessing liver stiffness using real-time shear wave elastography with an Aixplorer (SuperSonic Imagine, Aix-en-Provence, France) or transient elastography with the usage of FibroScan (Echosens, France). Based on the METAVIR score, according to the European Association for the Study of the Liver guidelines, the cutoff value of 13 kPa was used for the prediction of individuals with F4 who were considered to be cirrhotic[20]. The patients were scored on Child-Pugh (CP) and model for end-stage liver disease, and data on the presence of esophageal varices, past or present hepatic decompensation and the history of HCC, and liver transplantation were collected. Patients who scored as B or C on the CP scale were considered decompensated.

Efficacy assessment

The efficacy endpoint of the study was SVR. It was defined as undetectable HCV RNA at least 12 wk after completion of treatment. Patients with detectable HCV RNA at this time point were identified as virologic non-responders, whereas those with no HCV RNA assessment 12 wk after the end of treatment were considered lost to follow-up. Depending on local practices at the testing site, the concentration of HCV RNA was measured using COBAS TaqMan HCV v2.0 (Roche Molecular Diagnostics, Pleasanton, CA, United States), COBAS AmpliPrep HCV (Roche Molecular Diagnostics, Pleasanton, CA, United States), the m2000 Real-Time System (Abbott Molecular, Des Plaines, IL, United States), or the Xpert HCV Viral Load real-time assay (Cepheid, Sunnyvale, California, United States).

Safety assessment

Through treatment and 12 wk after its completion, the following safety data were collected: The occurrence of adverse events (AEs), including severe AEs, and death, as well as the rates of modification or discontinuation of the therapy course. In addition, AEs of special interest related to the deterioration of liver function involving gastrointestinal bleeding, ascites, and encephalopathy were reported.

Ethics

The data were originally collected not for scientific purposes but to evaluate treatment efficacy and safety in real-world settings with registered medications. Patients were not exposed to any experimental interventions. According to the local law (the Polish Pharmaceutical Law of 6 September 2001, art. 37al), noninterventional studies do not require ethics committee approval. Due to the retrospective design of the analysis, additional consent from patients was not required, but as mentioned, they signed a consent form to enter the therapeutic program. Data of patients were collected and analyzed following applicable data protection rules.

Statistical analysis

Categorical data are presented as numbers and percentages, whereas continuous data are expressed as the mean (SD) or median and interquartile ranges. The SVR was evaluated for all patients who initiated the treatment after the exclusion of those lost to follow-up as per protocol analysis. Statistical analyses were performed using Statistica v. 13 (StatSoft, Tulsa, OK, United States). Multiple logistic regression was used to predict the odds of no response to HCV treatment based on predictor variables selected through univariate analysis.

RESULTS

Patient characteristics

The studied population consisted of 3577 patients selected from 14801 individuals treated with IFN-free regimens included in the EpiTer-2 database. They were divided into six groups based on the date of treatment initiation: (1) July 1, 2015 to December 31, 2016; (2) January 1, 2017 to December 31, 2017; (3) January 1, 2018 to December 31, 2018; (4) January 1, 2019 to December 31, 2019; (5) January 1, 2020 to

December 31, 2020; and (6) January 1, 2021 to December 31, 2021 (Figure 1).

Between the first period analyzed and 2018, the percentage of cirrhotic patients treated with antiviral therapy significantly decreased from 43.7% to 14.8%, but they began to rise again in 2019 to a value of 21.3% in 2021. The analyzed population was sex-balanced until 2017, while men's predominance was observed for consecutive years. Between the first and the last time interval, a minor reduction in the median (Q1, Q3) age was documented in women: From 63 (57, 69) in 2015-2016 to 61 (53, 68.5) years in 2021 and slightly greater reduction in men age: From 57 (47, 63) in 2015-2016 to 52 (44, 63) years in 2021 (Table 1). Women were older than men, with a peak of around 60-70 years in all the analyzed periods (Figure 2).

Age distribution in men demonstrated the first peak around age 56 to 65 and the second around age 41 to 45. The first peak was dominant until 2019, whereas the second peak appeared in 2018 and was higher in 2020 and 2021. The patient's body mass index (BMI) was comparable irrespective of the analyzed time interval (Table 1). The prevalence of comorbidities initially decreased from 76.8% in 2015-2016 to 69.2% in 2019, but increased again in 2020 and 2021 (70.9% and 73.9%, respectively). The proportion of the most frequent comorbidities of hypertension and diabetes ranged from 36.2% in 2020 to 48.7% in 2018 and from 17.4% in 2019 to 27% in 2021, respectively. The rate of patients using concomitant medications decreased from 76.8% in 2017 to 69.3% in 2020, with a slight increase to 71.8% in 2021.

Characteristics of HCV infection and liver disease severity

GT1b was the most common in all periods, but a reduction in the prevalence of infections with this GT from 89.2% to 62.8% between 2015 and 2020 was documented (Table 2). A renewed increase in the share of GT1b infection to 72.4% was noted in the last time frame analyzed. In parallel with the decrease in the percentage of GT1b, an increase in the share of GT3 was observed, ranging from 4.7% in 2015-2016 to 30.2% in 2020. Analogically to GT1b, a reversal of the earlier trend was documented in the last period. The percentage of patients classified as CP classes B and C, which was 10.5% in the first period analyzed, initially showed a downward trend, eventually increasing to 15.2% in the last time interval. No tendency was observed in the incidence of a history of hepatic decompensation, HCC, HBV, and HIV coinfection, as well as the presence of decompensation at baseline. Documented esophageal varices and a history of liver transplantation were more commonly observed at the beginning of the IFN-free era.

Treatment characteristics

There has been a strong and steady trend of an increasing share of treatment-naïve patients over the years (Table 3). At the beginning of the IFN-free era, the majority of those with previous treatment failure had been previously treated with PegIFN ± ribavirin (RBV), and this tendency was observed until 2018. Since 2019, two-thirds of individuals were retreated after the failure of IFN-free treatment. From 2015 to 2018, the most common therapeutic options were GT-specific regimens, particularly ombitasvir (OBV)/paritaprevir (PTV)/[ritonavir (r) ± dasabuvir (DSV) ± RBV] in 2015-2017 and grazoprevir (GZR)/[elbasvir (EBR) ± RBV] in 2018. In subsequent years, these regimens were replaced by pangenotypic options, which were used to treat about 90% of patients as of 2020. In 2019 and 2020, the glecaprevir (GLE)/pibrentasvir (PIB) combination was the most commonly used regimen, while in the last interval, most patients were treated with sofosbuvir (SOF)/[velpatasvir (VEL) ± RBV].

Treatment effectiveness

The overall SVR after excluding lost-to-follow-up patients was 95% (Table 4). A slightly higher effectiveness of 97.8% was noted in patients treated with the most common option of OBV/PTV/(r ± DSV ± RBV). A similar, high virologic response rate was achieved with other GT-specific regimens [LDV/(SOF ± RBV), GZR/(EBR ± RBV), and pangenotypic GLE/PIB combinations]. The administration of another pangenotypic option, VEL/(SOF ± RBV), resulted in a 91.6% cure rate. The lowest SVR rates were observed in those treated with SOF + RBV and asunaprevir (ASV) + daclatasvir (DCV) regimens - 72.9% and 85.5%, respectively. The smallest subgroups of patients treated with GLE/PIB/(SOF + RBV), SOF + (DCV ± RBV), SOF + (simeprevir ± RBV), and voxilaprevir (VOX)/VEL/SOF achieved a 100% treatment response.

Treatment effectiveness remained nearly similar in all six analyzed time intervals (Figure 3A). Analysis of SVR by GT showed comparable rates for all periods analyzed. The lowest response rates were obtained in GT3-infected patients (Figure 3C). A statistically significant higher SVR was observed in women between the first period analyzed and 2018 (Figure 3B). Univariate analysis showed that patients who did not achieve a virological response were significantly older, were more likely to be male, treatment-experienced, and had a higher BMI (Table 5).

The non-responding patient population also had a higher percentage of patients with GT3 infection, decompensated liver disease (CP classes B and C), and the presence of esophageal varices and liver decompensation in the form of encephalopathy at baseline. Those not responding virologically to treatment were significantly more likely to receive the ASV + DCV and SOF/(VEL ± RBV) regimens. A statistically significant difference in platelet count, bilirubin, and albumin levels between responders and virologic non-responders was also documented. Independent negative predictors of SVR in the

Table 1 Baseline characteristics of 3577 patients with cirrhosis treated with interferon-free regimens

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
Gender, females/males, *n* (%)	600 (50)/599 (50)	401 (48.5)/426 (51.5)	251 (43.8)/322 (56.2)	164 (37.4)/274 (62.6)	71 (35.7)/128 (64.3)	140 (41.1)/201 (58.9)
Age (yr), mean ± SD; min-max	58.6 ± 11.8; 21-97	59.4 ± 12.0; 22-87	58.2 ± 13.8; 21-89	55.2 ± 13.2; 26-91	54.6 ± 12.1; 28-83	56.6 ± 12.6; 25-86
Females	62.2 ± 10.8; 25-91	62.1 ± 11; 25-85	61.8 ± 14.2; 21-89	60.3 ± 12.8; 30-91	59.4 ± 12.2; 29-83	60.5 ± 12.3; 25-85
Males	55.1 ± 11.8; 21-97	56.8 ± 12.3; 22-87	55.4 ± 12.8; 27-88	52.1 ± 12.5; 26-89	51.9 ± 11.2; 28-80	53.9 ± 12.1; 30-86
Median (Q1, Q3)	60 (52, 67)	60 (52, 67)	59 (48, 68)	56 (45, 63)	55 (45, 63)	57 (47, 65)
Females, median (Q1, Q3)	63 (57, 69)	62 (56, 70)	62 (54, 73)	61 (52.5, 68)	60 (52, 68)	61 (53, 68.5)
Males, median (Q1, Q3)	57 (47, 63)	57 (48, 65)	56 (45, 64)	51 (42, 61)	52 (44, 60)	52 (44, 63)
BMI, mean ± SD; min-max	27.3 ± 4.5; 13.4-49.4	27.5 ± 4.8; 14.2-45.5	27.1 ± 4.7; 21-89	27.7 ± 5.2; 17-52.5	27.9 ± 5.6; 16-57.4	27.5 ± 4.9; 15.6-50
Comorbidities, *n* (%)						
Any comorbidity	921 (76.8)	682 (82.5)	431 (75.2)	303 (69.2)	141 (70.9)	252 (73.9)
Hypertension	582 (48.5)	384 (46.4)	279 (48.7)	179 (40.9)	72 (36.2)	158 (46.3)
Diabetes	263 (21.9)	212 (25.6)	120 (20.9)	76 (17.4)	44 (22.1)	136 (27)
Renal disease	48 (4)	39 (4.7)	20 (3.5)	10 (2.3)	9 (4.5)	12 (3.5)
Autoimmune diseases	29 (2.4)	14 (1.7)	8 (1.4)	9 (2.1)	2 (1)	3 (0.9)
Non-HCC tumors	20 (1.7)	15 (1.8)	12 (2.1)	12 (2.7)	6 (3)	15 (4.4)
Other	628 (52.4)	560 (67.7)	352 (61.4)	208 (47.5)	147 (73.9)	179 (52.5)
Concomitant medications, *n* (%)	879 (73.3)	635 (76.8)	431 (75.2)	305 (69.6)	138 (69.3)	245 (71.8)
ALT IU/L, mean ± SD	97.3 ± 66.2	96.4 ± 69.9	96 ± 85	107.1 ± 91.7	114.5 ± 103.8	115.9 ± 101.2
Bilirubin mg/dL, mean ± SD	1.2 ± 0.9	1 ± 0.7	1.1 ± 0.9	1.1 ± 0.9	1.1 ± 1.6	1.1 ± 0.9
Albumin g/dL, mean ± SD	5.8 ± 12.8	3.8 ± 0.5	3.8 ± 0.5	3.9 ± 0.5	3.8 ± 0.5	3.9 ± 0.6
Creatinine mg/dL, mean ± SD	1.1 ± 3.8	0.8 ± 0.5	0.9 ± 0.5	0.9 ± 0.7	0.9 ± 0.3	0.9 ± 0.7
Hemoglobin g/dL, mean ± SD	14.1 ± 1.8	14.1 ± 1.8	14 ± 1.8	14.1 ± 1.9	14.2 ± 1.8	13.9 ± 1.9
Platelets, × 1000/μL, mean ± SD	118.7 ± 61.6	136.1 ± 67.9	135 ± 70	136.5 ± 61.9	145.8 ± 87.6	145.6 ± 76.9
HCV RNA × 10^6 IU/mL, mean ± SD	1.6 ± 4.6	2.5 ± 6.4	2 ± 4.8	3.3 ± 24	1.7 ± 2.6	2 ± 3.2

IFN: Interferon; SD: Standard deviation; BMI: Body mass index; HCC: Hepatocellular carcinoma; ALT: Alanine transaminase; HCV RNA: Ribonucleic acid of hepatitis C virus.

logistic regression analysis were male gender [odds ratio (OR) = 2.28], GT3 infection (OR = 6.4), and prior failed treatment (OR = 1.66), while OBV/PTV/(r ± DSV ± RBV) therapy significantly increased the chance of response (OR = 0.58) (Table 6).

Treatment safety

As shown in Table 7, increasing tolerability was observed over time. The higher percentage of patients who completed a course of therapy as scheduled, from 89.5% (in 2015-2016) to 97.9% (in 2021), was assisted by a decreasing incidence of AEs, including serious ones, those leading to treatment discontinuation, and deaths. The most commonly reported AEs were mild, and weakness or fatigue remained the most frequent in all subsequent time intervals. The improvement in safety profile was accompanied by a decrease in RBV use from 71.3% in 2015-2016 to 6.7% in 2021. None of the 45 deaths were reported by the treating physician as related to antiviral therapy.

Table 2 Characteristics of liver disease in six-time intervals

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
GT, *n* (%)						
1	17 (1.4)	22 (2.7)	15 (2.6)	7 (1.6)	1 (0.5)	7 (2.1)
1a	21 (1.8)	17 (2.1)	10 (1.7)	10 (2.3)	9 (4.5)	8 (2.3)
1b	1070 (89.2)	652 (78.8)	420 (73.3)	291 (66.4)	125 (62.8)	247 (72.4)
2	0	0	1 (0.2)	3 (0.7)	0	3 (0.9)
3	56 (4.7)	103 (12.4)	109 (19.0)	111 (25.3)	60 (30.2)	63 (18.5)
4	35 (2.9)	33 (4.0)	17 (3)	16 (3.7)	4 (2)	13 (3.8)
5	0	0	0	0	0	0
6	0	0	1 (0.2)	0	0	0
Child-Pugh class, *n* (%)						
A	1033 (86.2)	724 (87.6)	521 (90.9)	397 (90.7)	176 (88.4)	285 (83.6)
B	118 (9.8)	77 (9.3)	47 (8.2)	36 (8.2)	18 (9.1)	49 (14.3)
C	9 (0.7)	1 (0.1)	2 (0.4)	1 (0.2)	1 (0.5)	3 (0.9)
No data	39 (3.3)	25 (3.0)	3 (0.5)	4 (0.9)	4 (2.0)	4 (1.2)
MELD score, *n* (%)						
< 15	1072 (89.4)	757 (91.5)	529 (92.3)	414 (94.5)	188 (94.5)	319 (93.5)
15-18	45 (3.8)	28 (3.4)	29 (5.1)	12 (2.8)	3 (1.5)	14 (4.1)
19-20	11 (0.9)	12 (1.5)	5 (0.9)	4 (0.9)	2 (1)	4 (1.2)
> 20	13 (1.1)	4 (0.5)	7 (1.2)	3 (0.7)	2 (1)	2 (0.6)
No data	58 (4.8)	26 (3.1)	3 (0.5)	5 (1.1)	4 (2)	2 (0.6)
History of hepatic decompensation, *n* (%)						
Ascites	124 (10.3)	65 (7.8)	64 (11.2)	26 (5.9)	15 (7.5)	47 (13.8)
Encephalopathy	33 (2.8)	15 (1.8)	19 (3.3)	4 (0.9)	2 (1)	7 (2.1)
Documented esophageal varices, *n* (%)	375 (31.3)	202 (24.4)	127 (22.2)	85 (19.4)	38 (19.1)	58 (17)
Hepatic decompensation at baseline, *n* (%)						
Moderate ascites-responded to diuretics	47 (3.9)	30 (3.6)	29 (5.1)	22 (5)	10 (5)	29 (8.5)
Tense ascites-not responded to diuretics	3 (0.3)	0	2 (0.3)	0	0	3 (0.9)
Encephalopathy	30 (2.5)	13 (1.6)	7 (1.2)	7 (1.6)	2 (1)	9 (2.6)
HCC history, *n* (%)	57 (4.8)	30 (3.6)	19 (3.3)	15 (3.4)	7 (3.5)	19 (5.6)
OLTx history, *n* (%)	23 (1.9)	4 (0.5)	1 (0.2)	1 (0.2)	0	1 (0.3)
HBV coinfection (HBsAg+), *n* (%)	12 (1)	12 (1.5)	12 (2.1)	8 (1.8)	3 (1.5)	6 (1.8)
HIV coinfection, *n* (%)	13 (1.1)	16 (1.9)	16 (2.8)	28 (6.4)	9 (4.5)	10 (2.9)

GT: Genotype; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; OLTx: Orthotopic liver transplantation; HBV: Hepatitis B virus; HBsAg+: Hepatitis B surface antigen; HIV: Human immunodeficiency virus.

DISCUSSION

The WHO has established the goal of eliminating HCV as a significant public threat by 2030, defined as a 90% reduction in new chronic infections and a 65% reduction in mortality compared to 2015[1]. In Poland, in middle-2015, the introduction of a therapeutic regimen program of IFN-free regimens for patients with CHC gave hope that the WHO goal could be achieved in our country. The key to reducing

Table 3 Treatment characteristics in six-time intervals

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
History of previous therapy						
Treatment-naïve	543 (45.3)	532 (64.3)	470 (82.0)	385 (87.9)	171 (86)	318 (93.2)
Nonresponder	290 (24.2)	103 (12.5)	23 (4.0)	7 (1.6)	6 (3)	11 (3.2)
Relapser	173 (14.4)	72 (8.7)	49 (8.5)	35 (8)	17 (8.5)	6 (1.8)
Discontinuation due to safety reason	71 (5.9)	40 (4.8)	14 (2.5)	4 (0.9)	1 (0.5)	3 (0.9)
Unknown type of response	120 (10.0)	76 (9.2)	14 (2.5)	7 (1.6)	2 (1)	3 (0.9)
No data	2 (0.2)	4 (0.5)	3 (0.5)	0	2 (1)	0
Number of patients with treatment failure	n = 654	n = 291	n = 100	n = 53	n = 26	n = 23
IFN ± RBV	64 (9.8)	36 (12.4)	2 (2)	0	0	0
PegIFN ± RBV	390 (59.6)	197 (67.7)	48 (48)	14 (26.4)	9 (34.6)	9 (39.1)
PegIFN + RBV + DAA	196 (30)	47 (16.1)	10 (10)	3 (5.7)	0	0
IFN-free	4 (0.6)	11 (3.8)	39 (39)	35 (66)	17 (65.4)	14 (60.9)
No data	0	0	1 (1)	1 (1.9)	0	0
Current treatment regimen						
Genotype-specific treatment regimens						
ASV + DCV	42 (3.5)	13 (1.6)	0	0	0	0
LDV/(SOF ± RBV)	350 (29.2)	283 (34.2)	154 (26.9)	16 (3.7)	5 (2.5)	4 (1.2)
OBV/PTV/(r ± DSV ± RBV)	745 (62.1)	327 (39.5)	32 (5.6)	0	0	1 (0.3)
GZR/(EBR ± RBV)	0	97 (11.7)	205 (35.8)	76 (17.3)	16 (8)	15 (4.4)
Other	62 (5.2)	104 (12.6)	19 (3.3)	0	0	0
SOF ± SMV ± DCV ± RBV, SMV ± DCV ± RBV						
Pangenotypic regimens						
GLE/PIB	0	1 (0.1)	59 (10.3)	178 (40.6)	99 (49.8)	117 (34.3)
GLE/(PIB + SOF + RBV)	0	0	0	2 (0.5)	0	0
SOF/(VEL ± RBV)	0	2 (0.3)	104 (18.1)	166 (37.9)	79 (39.7)	195 (57.2)
VOX/VEL/SOF	0	0	0	0	0	9 (2.6)
Current-RBV-containing therapies	855 (71.3)	360 (43.5)	163 (28.4)	44 (10)	12 (6)	23 (6.7)

IFN: Interferon; RBV: Ribavirin; PegIFN: Pegylated interferon; DAA: Direct-acting antivirals; ASV: Asunaprevir; DCV: Daclatasvir; LDV: Ledipasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; GZR: Grazoprevir; EBR: Elbasvir; SMV: Simeprevir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; VOX: Voxilaprevir.

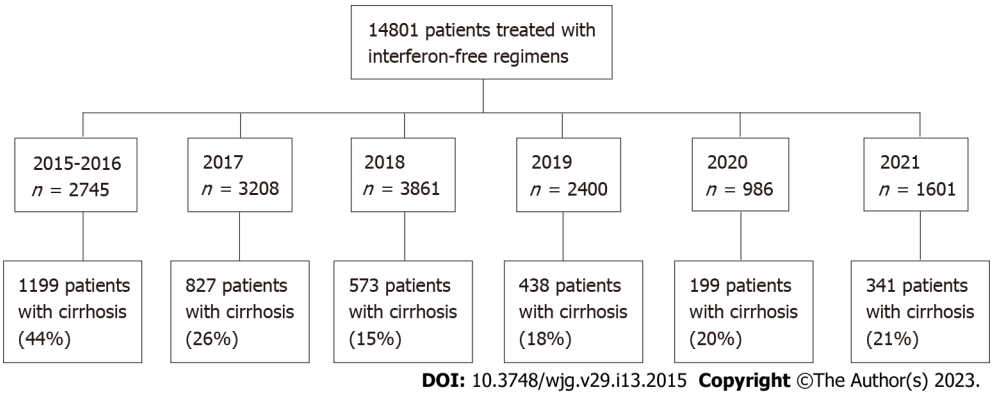
mortality was using antiviral treatment in patients with the most advanced liver disease, as this population is most at risk of death[21]. It should be mentioned at this point that some countries initially reimbursed the therapy only for patients with advanced fibrosis and cirrhosis[22].

Despite the lack of restrictions in the Polish drug program, at the beginning of the IFN-free era, cirrhotic patients had priority in access to treatment due to long waiting lists. In the current study, they accounted for almost 44% of all treated patients in 2015-2016. This percentage decreased in consecutive periods, but the trend has been reversed since 2019. In the latter two intervals, not only were patients with cirrhosis reported more frequently, but this was also accompanied by an increase in the share of those with liver decompensation. One possible reason for the increase in cirrhosis rates is newly diagnosed cases in advanced stages of the disease in patients previously unaware of HCV infection[23]. This hypothesis is supported by the increasing percentage of patients previously untreated. At the same time, the total number of treated patients decreased. Other researchers point to the negative impact of

Table 4 Treatment effectiveness according to regimen, calculated as per protocol analysis

Regimen	SVR PP, *n* (%)
All regimens	3271/3442 (95)
OBV/PTV/(r ± DSV ± RBV)	1052/1076 (97.8)
LDV/(SOF ± RBV)	748/778 (96.1)
GZR/(EBR ± RBV)	387/398 (97.2)
VEL/(SOF ± RBV)	477/521 (91.6)
GLE/PIB	420/435 (96.6)
GLE/PIB/(SOF + RBV)	2/2 (100)
ASV + DCV	47/55 (85.5)
SOF + DCV ± RBV	21/21 (100)
SOF + RBV	105/144 (72.9)
SOF + SMV ± RBV	5/5 (100)
VOX/VEL/SOF	7/7 (100)

PP: Per protocol; SVR: Sustained virological response; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; RBV: Ribavirin; LDV: Ledipasvir; SOF: Sofosbuvir; GZR: Grazoprevir; EBR: Elbasvir; VEL: Velpatasvir; GLE: Glecaprevir; PIB: Pibrentasvir; ASV: Asunaprevir; DCV: Daclatasvir; SMV: Simeprevir; VOX: Voxilaprevir.



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Figure 1 Distribution of patients treated for hepatitis C virus infection in six-time intervals.

the coronavirus disease 2019 (COVID-19) pandemic on the number of diagnosed and treated patients with chronic HCV infection[24,25].

To the best of our knowledge, this is the first study to track changes in patient profiles, therapeutic options used, and their efficacy in patients with cirrhosis in the IFN-free era. Our work summarizes 7 years of access to IFN-free therapy in Poland. In the current study, a slight decrease in the age of patients with cirrhosis was observed between the first and last time interval. This reduction was much less pronounced compared to analyzes of demographic changes in CHC patients regardless of liver fibrosis[11,13]. The differences can be explained by the fact that the cirrhotic patient population tended to be older compared to patients with non-advanced liver fibrosis. The age distribution was irregular and evolved throughout the entire analyzed period. Although the two-peak pattern was described previously not only in the Polish population with CHC, we noted only one peak at ages 60 to 70 in women in all six-time intervals. We observed a second peak around the age of 41 to 45, only in men in 2018-2021[11,13,26].

In addition to prioritizing cirrhotic patients at the beginning of the IFN-free era, those after previous treatment failure were also given priority in access to therapy. This explains the decreasing percentage of treatment-experienced patients over time, and it should be emphasized that despite the COVID-19 pandemic in 2020 and 2021, this trend continued. Patients after previous ineffective antiviral treatment up to 2018 were more likely to be on failed IFN-based regimens, while 2019-2021 documented a higher proportion of patients on failed DAA therapy, which is understandable, given the evolution and availability of antiviral regimens. An additional factor contributing to the growing proportion of treatment-naïve individuals was the detection of HCV infection in patients previously unaware of it.

Table 5 Comparison of responders and virological non-responders to antiviral therapy

Parameter	Responders (*n* = 3271)	Non-responders (*n* = 171)	*P* value
Gender, females/males, *n* (%)	1539 (47)/1732 (53)	42 (24.6)/129 (75.4)	< 0.0001
Age (yr), mean ± SD; min-max	58.0 ± 12.6; 21-97	55.0 ± 10.5; 29-84	0.0002
Females	61.6 ± 11.8; 21-91	59.9 ± 10.6; 35-81	0.3173
Males	54.8 ± 12.4; 21-97	53.4 ± 10; 29-84	0.1045
BMI, mean ± SD; min-max	27.4 ± 5.0; 13.4-57.4	28.7 ± 4.6; 16-47.5	0.0001
Current treatment regimen			
Genotype-specific treatment regimens			
ASV + DCV	47 (1.4)	8 (4.7)	0.0001
LDV/(SOF ± RBV)	748 (22.9)	30 (17.5)	0.1047
OBV/PTV/(r ± DSV ± RBV)	1052 (32.2)	24 (14)	< 0.0001
GZR/(EBR ± RBV)	387 (11.8)	11 (6.4)	0.0314
Pangenotypic regimens			
GLE/PIB	420 (12.8)	15 (8.8)	0.1186
GLE/(PIB + SOF + RBV)	2 (0.1)	0	0.7464
SOF/(VEL ± RBV)	477 (14.6)	44 (25.7)	0.0001
VOX/VEL/SOF	7 (0.2)	0	0.5448
GT, *n* (%)			< 0.0001
1	63 (1.9)	2 (1.2)	
1a	70 (2.2)	1 (0.6)	
1b	2624 (80.2)	89 (52.0)	
3	397 (12.1)	76 (44.4)	
4	110 (3.4)	3 (1.8)	
Other	7 (0.2)	0	
Comorbidities, *n* (%)			
Any comorbidity	2498 (76.4)	131 (4)	0.9425
Hypertension	1533 (46.9)	66 (2)	0.0345
Diabetes	731 (22.3)	46 (1.4)	0.1651
Renal disease	124 (3.8)	8 (0.2)	0.5558
Autoimmune diseases	63 (1.9)	0	0.0670
Non-HCC tumors	68 (2.1)	6 (0.2)	0.1963
Other	1783 (54.5)	96 (2.9)	0.6762
Concomitant medications, *n* (%)	2389 (73.0)	136 (79.5)	0.0610
Treatment experienced	1039 (31.8)	69 (40.4)	0.0208
History of hepatic decompensation, *n* (%)			
Ascites	297 (9.0)	21 (12.2)	0.1588
Encephalopathy	65 (2.0)	7 (4.1)	0.0606
Documented esophageal varices, *n* (%)	780 (23.8)	64 (37.4)	0.0001
Hepatic decompensation at baseline, *n* (%)	173 (5.3)	18 (10.5)	0.0035
HCC history, *n* (%)	117 (3.6)	9 (5.3)	0.2523
OLTx history, *n* (%)	28 (0.9)	1 (0.6)	0.7052
Child-Pugh class, *n* (%)			

B or C	296 (9)	29 (17)	0.0006
HBV coinfection (HBsAg+), *n* (%)	50 (1.5)	2 (1.2)	0.7075
HIV coinfection, *n* (%)	79 (2.4)	6 (3.5)	0.3690
ALT IU/L, mean ± SD	101.2 ± 80.8	106.9 ± 82.0	0.3385
Bilirubin mg/dL, mean ± SD	1.1 ± 0.9	1.2 ± 0.7	< 0.0001
Albumin g/dL, mean ± SD	4.5 ± 7.7	3.9 ± 3.1	0.0002
Creatinine mg/dL, mean ± SD	0.9 ± 2.3	0.8 ± 0.2	0.8562
Hemoglobin g/dL, mean ± SD	14.1 ± 1.8	13.9 ± 1.8	0.1319
Platelets, × 1000/μL, mean ± SD	132.6 ± 66.8	109.2 ± 59.4	< 0.0001
HCV RNA × 10^6 IU/ml, mean ± SD	2.2 ± 10.0	2.0 ± 2.9	0.1123

SD: Standard deviation; BMI: Body mass index; ASV: Asunaprevir; DCV: Daclatasvir; LDV: Ledipasvir; SOF: Sofosbuvir; RBV: Ribavirin; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; GZR: Grazoprevir; EBR: Elbasvir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; VOX: Voxilaprevir; GT: Genotype; HCC: Hepatocellular carcinoma; OLTx: Orthotopic liver transplantation; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HIV: Human immunodeficiency virus; ALT: Alanine transaminase; HCV RNA: Ribonucleic acid of hepatitis C virus.

Table 6 Logistic multiple regression results on association between selected parameters significant in univariate analysis and non-response to antiviral treatment

Parameter	OR	95%CI	*P* value
Age ≥ 75 yr	0.81	0.38-1.72	0.5915
Obesity (BMI ≥ 30 kg/m^2)	1.15	0.80-1.66	0.4473
Male gender	2.28	1.57-3.29	< 0.0001
Platelets < 100000/μL	1.37	0.98-1.93	0.0694
GT1b	1.54	0.66-3.59	0.3145
GT3	6.40	2.69-15.22	< 0.0001
Bilirubin > 3 mg/dL	0.29	0.08-1.00	0.0506
Albumin < 2.8 g/dL	0.98	0.44-2.20	0.966
Documented esophageal varices	1.43	1.00-2.06	0.052
Hepatic decompensation at baseline	1.49	0.76-2.91	0.2496
Hypertension	0.93	0.67-1.30	0.6783
Child-Pugh, B or C	1.80	0.95-3.42	0.0716
SOF/(VEL ± RBV)	1.13	0.76-1.69	0.541
GZR/(EBR ± RBV)	0.91	0.46-1.80	0.7951
OBV/PTV/(r ± DSV ± RBV)	0.58	0.35-0.95	0.0319
Treatment experienced	1.66	1.18-2.33	0.0039

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; GT: Genotype; SOF: Sofosbuvir; VEL: Velpatasvir; RBV: Ribavirin; GZR: Grazoprevir; EBR: Elbasvir; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir.

Since DAA therapy lasts much shorter than previously used IFN-based regimens, in the absence of restrictions on treatment reimbursement, the waiting list was shortened considerably and subsequently ceased to exist, while antiviral therapy is implemented immediately after the diagnosis is established [27]. This makes the characteristics of newly diagnosed patients quickly change the profile of the patients treated.

There was also a significant change in the share of HCV GTs in the treated patients. The most common HCV GT in Poland was GT1b[28,29]. Our study restricted to individuals with cirrhosis confirmed these findings, but we noted a changing distribution of GTs over time depending on the availability of treatment regimens in subsequent years. At the beginning of the IFN-free era, GT1- and GT4-infected patients had access to highly effective GT-specific regimens, while patients with GT3

Table 7 Safety of antiviral therapy in six-time intervals

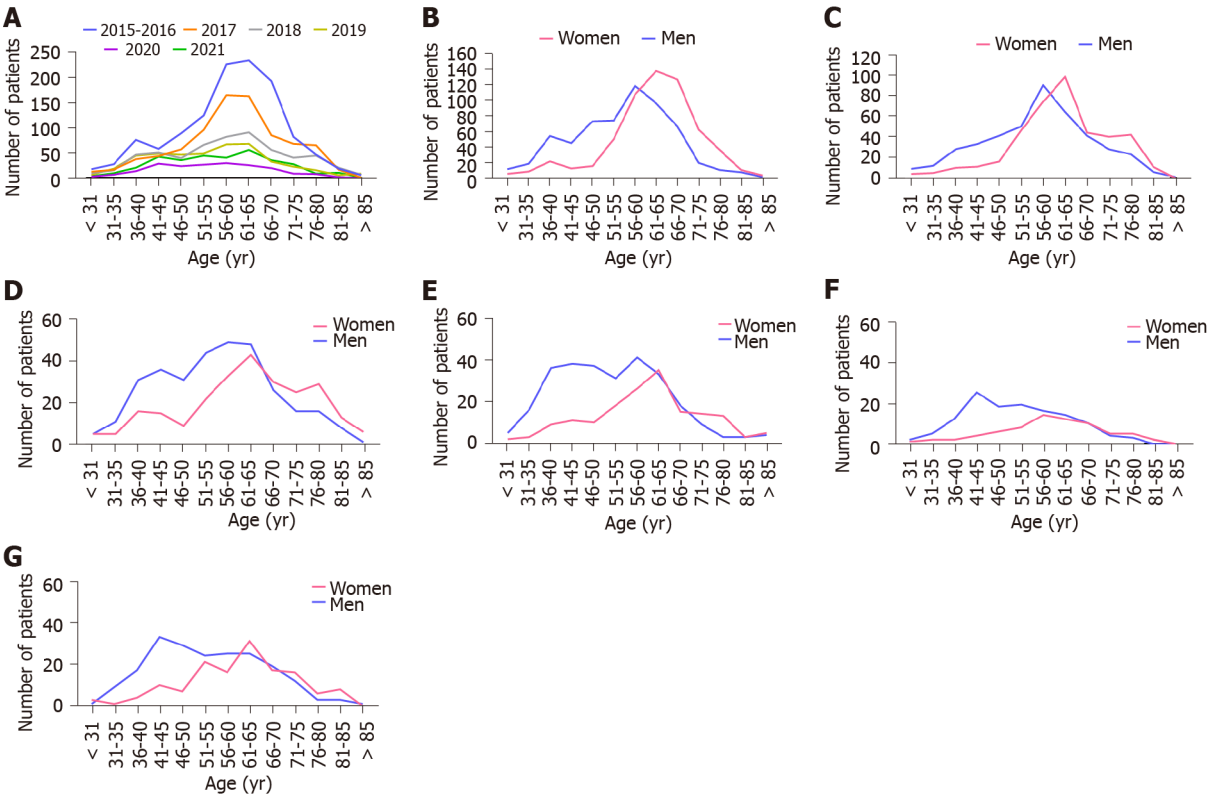
Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
Treatment course, *n* (%)						
According to schedule	1073 (89.5)	776 (93.8)	538 (93.9)	428 (97.7)	196 (98.5)	334 (97.9)
Therapy modification	71 (5.9)	21 (2.6)	26 (4.5)	2 (0.5)	0	0
Therapy discontinuation	44 (3.7)	19 (2.3)	9 (1.6)	5 (1.1)	1 (0.5)	6 (1.8)
No data	11 (0.9)	11 (1.3)	0	3 (0.7)	2 (1.0)	1 (0.3)
Patients with at least one AE, *n* (%)	500 (41.7)	212 (25.6)	139 (24.3)	80 (18.3)	36 (18.1)	66 (19.4)
Serious adverse events, *n* (%)	48 (4)	11 (1.3)	16 (2.8)	7 (1.6)	4 (2)	3 (0.9)
AEs leading to treatment discontinuation, *n* (%)	35 (2.9)	16 (1.9)	4 (0.7)	2 (0.5)	1 (0.5)	1 (0.3)
Most common AEs (≥ 2%), *n* (%)						
Weakness/fatigue	242 (20.2)	78 (9.4)	59 (10.3)	28 (6.4)	13 (6.5)	16 (4.7)
Anemia	63 (5.3)	36 (4.4)	17 (3)	2 (0.5)	0	2 (0.6)
Sleep disorders	52 (4.3)	20 (2.4)	20 (3.5)	4 (0.9)	6 (3)	6 (1.8)
Nausea	32 (2.7)	14 (1.7)	3 (0.5)	1 (0.2)	5 (2.5)	6 (1.8)
Abdominal pain	21 (1.8)	7 (0.8)	4 (0.7)	12 (2.7)	5 (2.5)	8 (2.3)
Headaches	50 (4.2)	14 (1.7)	11 (1.9)	7 (1.2)	3 (0.5)	8 (2.3)
Myalgia/arthralgia	18 (1.5)	18 (2.2)	9 (1.6)	6 (1.4)	6 (3)	8 (2.3)
Pruritis	71 (5.9)	19 (2.3)	10 (1.7)	10 (2.3)	7 (3.5)	7 (2.1)
Skin lesions	26 (2.2)	14 (1.7)	7 (1.2)	5 (1.1)	2 (1)	4 (1.2)
Bilirubin, > ULN	44 (3.7)	30 (3.6)	3 (0.5)	3 (0.7)	0	0
AEs of particular interest						
Ascites	30 (2.5)	23 (2.8)	12 (2.1)	6 (1.4)	0	13 (3.8)
Hepatic encephalopathy	28 (2.3)	12 (1.5)	6 (1.0)	2 (0.5)	1 (0.5)	6 (1.8)
Gastrointestinal bleeding	9 (0.8)	4 (0.5)	5 (0.9)	1 (0.2)	1 (0.5)	0
Death, *n* (%)	13 (1.1)	10 (1.2)	10 (1.7)	3 (0.7)	2 (1.0)	7 (2.1)

AE: Adverse event; ULN: Upper limit of normal.

infection in Poland were still treated with IFN-based options as the available SOF + RBV was considered suboptimal due to lower SVR[30,31]. The SOF + (DCV ± RBV) regimen with activity against GT3 was used incidentally in Poland due to problems with reimbursement. However, our analysis confirmed the high effectiveness of this option. For this reason, patients infected with GT, especially subtype 1b, definitely dominated in the initially analyzed time intervals. In 2018, new pangenotypic options, including GLE/PIB and SOF/(VEL ± RBV), which are highly effective for all HCV GTs, became available in Poland, thus enabling effective treatment of patients infected with GT3. This was reflected in the increase in the percentage of this population, despite the still visible dominance of GT1b resulting from the original distribution of GTs in the Polish population. Our findings are also supported by an RWE study conducted in Germany among patients with HCV infection, regardless of cirrhosis status treated with DAA[32].

Surprisingly, in the last analyzed period, we again recorded an increase in GT1b infections accompanied by a decrease in the percentage of GT3-infected patients. It seems that this change could have been influenced by both effective therapies of patients with GT3 infection, as well as the previously described phenomenon of detecting HCV infections in previously undiagnosed people, who, according to the original distribution of HCV GTs in the Polish population, are more likely to be infected with GT1b.

The change in the profile of patients with cirrhosis treated with DAA regimens in Poland was accompanied by changes in the options used related to the registration of new drugs and recommendations of the Polish Group of Experts for HCV[15-19]. At the beginning of the IFN-free era, GT-specific regimens were used, especially the OBV/PTV/(r + DSV ± RBV) combination, which was the first



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Figure 2 Age distribution in all patients treated in six-time intervals and according to gender in 2015-2016, 2017, 2018, 2019, 2020, and 2021. A: In six-time intervals; B: In 2015-2016; C: In 2017; D: In 2018; E: In 2019; F: In 2020; G: In 2021.

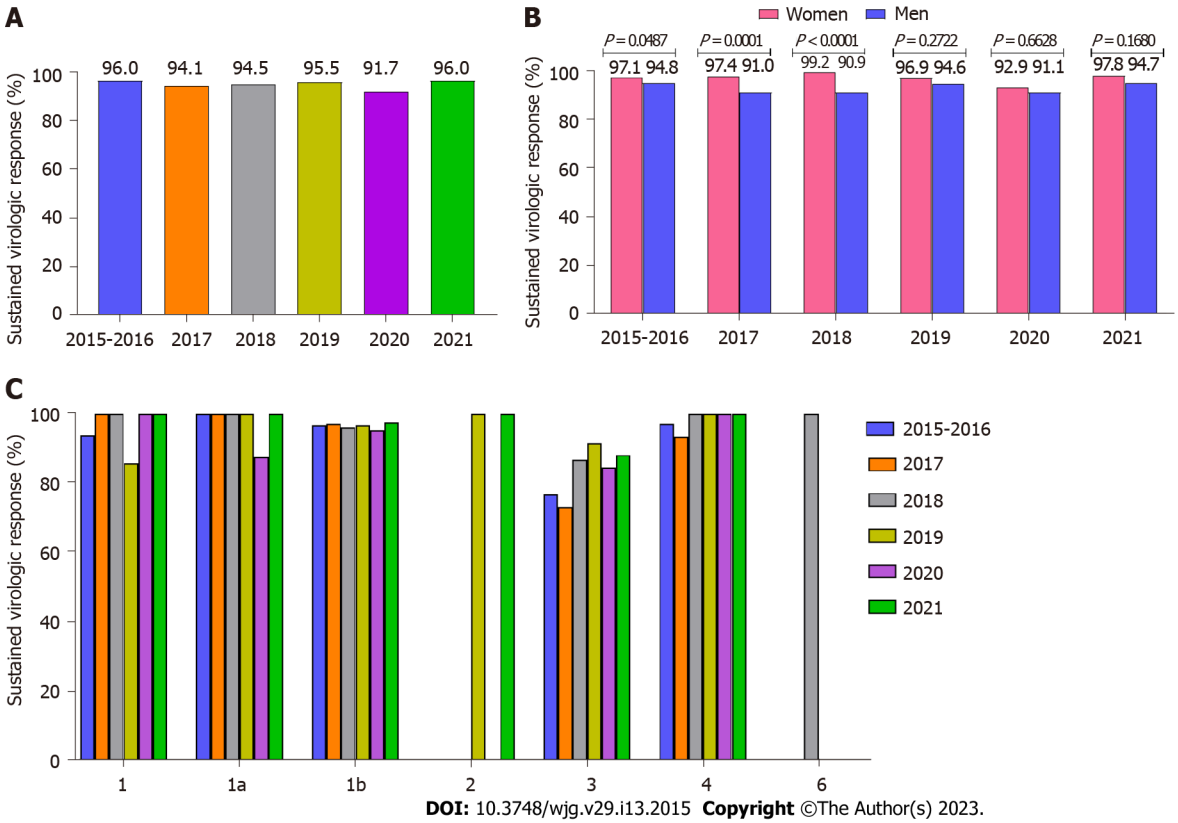
reimbursed IFN-free therapy in Poland. As mentioned above, as of 2018, pangenotypic regimens have become available in our country, and this has gradually displaced previous therapeutic options.

Regardless of the changing patient profile and therapeutic options, the effectiveness of therapy in this difficult-to-treat group of patients with advanced liver disease remained consistently high in all analyzed periods. The effectiveness of the administrated regimens was very high and exceeded 95% for most options. We documented the highest SVR for the regimen OBV/PTV/(r + DSV ± RBV), which was found to be an independent success factor in the multivariate analysis (OR = 0.58). This was an option used in the largest number of patients in the current study. Its efficacy of 99% in the population of patients with advanced liver disease was previously documented in the AMBER RWE study[33]. The high effectiveness achieved after treatment with other GT-specific regimens is also consistent with the results of the RWE GZR/(EBR ± RBV) and LDV/(SOF ± RBV) studies[34-36].

An excellent response rate of 97.6% was achieved in the GLE/PIB group, and these results were supported by a meta-analysis of 18 RWE studies[37]. Another pangenotypic regimen of VEL/(SOF ± RBV) was effective in 91.6%. Although the large meta-analysis from 12 cohorts documented a 97.9% SVR in 1078 patients with cirrhosis, it should be noted that only compensated patients were included in that analysis. In our cohort, 14% of patients receiving this option were decompensated, which may have affected the results obtained[38]. The lowest SVR rate of 72.9% was achieved by patients treated with SOF + RBV. This suboptimal therapy was received by individuals with GT3 infection who had contraindications or intolerance to PegIFN. Our results are lower than those demonstrated in the BOSTON study, where 24-wk SOF + RBV therapy in patients with GT3 cirrhosis was 79% effective[30]. On the other hand, the HCV-TARGET real-world study documented the effectiveness of this regimen in only 45% of patients with cirrhosis and GT3 infection[39].

GT3 infection was found in our study as an independent predictor of reduced SVR rate in multivariate analysis, in addition to male gender and history of prior therapy. Our observations are consistent with the results of a large retrospective RWE study involving 15720 United States veterans with a 40% share of cirrhotics, in whom GT3 infection significantly reduced the chances of responding to DAA[40]. However, it should be noted that in the above-cited study, SOF + RBV + PegIFN, LDV/(SOF + RBV) options were used in patients with GT3 infection, and only the introduction of highly effective pangenotypic options improved the response rate in this population[41].

Treatment experience has been documented as a factor associated with DAA therapy failure in the RWE study conducted by Chen *et al*[42]. In contrast, Berkan-Kawińska *et al*[43] did not find that it was a negative predictor of SVR among patients with cirrhosis. A specific subgroup of treatment-experienced patients is the population after the previous failure of DAA regimens. Despite other reports indicating a



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Figure 3 Sustained virological response rates by hepatitis C virus genotype, in six-time intervals (per protocol analysis), and according to gender. A: In six-time intervals (per protocol analysis); B: According to gender; C: By hepatitis C virus genotype.

lower cure rate in DAA-experienced cirrhotic patients, the POLARIS-1 and POLARIS-4 clinical trials showed that these patients retreated with the pangenotypic SOF/VEL/VOX combination achieved high efficacy of 93%-98%[44-46]. Although in the current analysis, the pangenotypic rescue option of SOF/VEL/VOX or GLE/(PIB + SOF + RBV) was used in only 11 patients after failure of therapy with DAA regimens, all achieved a virological response.

Similar to our results, male gender was an independent negative predictor of achieving SVR in a large study from the United States Veterans Affairs[40]. Data from other RWE studies confirmed these results [34,47]. The high effectiveness of DAA regimens in our analysis was accompanied by a good safety profile with a low rate of discontinuation due to AEs, which supports findings from clinical trials and other RWE studies[34,40,48-50]. Importantly, we observed an improvement in the safety profile over time, which is explained by shortening the therapy and reducing the frequency of using regimens containing RBV. Weakness/fatigue was the most common AE regardless of the analyzed period. The percentage of patients with symptoms of liver decompensation during therapy and the 12-wk follow-up period did not exceed a few percent, which proves the good safety profile of DAA in patients with cirrhosis and is consistent with the observations of other authors[51]. This is also confirmed by the overall death rate of 1.3%, varying from 0.7% to 2.1% over time.

Limitations

Our analysis has several limitations typical of retrospective studies, including possible physician bias due to incomplete data, inconsistent diagnosis or misclassification of data, underreporting of AEs, and possible data entry errors. In addition, the observational nature of the study may result in insufficient discipline during treatment; we did not capture data on adherence to the treatment, while available data suggest that lack of adherence to therapy may result in a lower chance of virologic response in cirrhotic patients treated with the SOF/LDV regimen[52,53].

Although the study was conducted in the same centers treating patients, and it seems that the decreasing overall number of patients and the changing percentage of patients with cirrhosis reflect the actual condition, distortion of the data by other factors cannot be excluded. One of these may be that the analysis also covered the COVID-19 pandemic period, when the availability of diagnostic tests and antiviral therapy was limited.

However, it is important to mention the main strength of our study, which is the collection of data from a truly geographically diverse population, representative of routine practice and a large number of patients enrolled from different centers in our country. This allows the generalization of the results. Another strong point is the large number of patients retained in post-treatment evaluation, with a rather

low rate of those lost to follow-up (2.5% after the exclusion of deaths). Notably, the current analysis is the first study to directly compare changes in patient profile, antiviral treatment characteristics, efficacy, and treatment safety in patients with cirrhosis over time in the era of IFN-free therapy.

CONCLUSION

The current analysis documents changes in the characteristics of patients with HCV-infected cirrhosis that have occurred over the seven years of access to IFN-free therapies in the form of a decline in the median age of patients, the prevalence of comorbidities, and the use of concomitant medications. In addition, the evolution of the therapeutic regimens used is described, in the course of which GT-specific options were supplanted by pangenotypic regimens. Regardless of these changes, patients achieved consistently high efficacy in all analyzed periods. Male gender, GT3 infection, and prior treatment were identified as independent negative predictors of SVR.

ARTICLE HIGHLIGHTS

Research background

Direct-acting antivirals (DAAs), which have replaced interferon (IFN)-based regimens, have significantly improved the prognosis of patients with cirrhosis, the population at highest risk for the most severe complications of infection with hepatitis C virus (HCV).

Research motivation

We aimed to track changes in the characteristics of HCV-infected patients with cirrhosis and document the evolving treatment regimens over the years, along with their efficacy and safety profile in this patient population.

Research objectives

Data of 3577 cirrhotics selected from 14801 HCV-infected patients treated between 2015 and 2021 with DAA regimens derived from the Epiter-2 database were analyzed.

Research methods

The analysis used demographic, clinical, and laboratory data of the studied population collected retrospectively in the Epiter-2 database. The measure of treatment effectiveness was the percentage of sustained virologic response (SVR) calculated after excluding patients lost to follow-up. Safety data collected during therapy and the 12-wk post-treatment period included information on adverse events, including serious ones, deaths, and treatment course.

Research results

From 2015 to 2017, the study population was gender-balanced, while male dominance was evident in subsequent years. The decrease in the median age of patients documented during the study was accompanied by a decrease in the percentage of patients with comorbidities and comedications. A steady increase in the percentage of treatment-naïve patients was observed over the years. The genotype (GT)-specific options dominant in 2015-2018 were then replaced by pangenotypic regimens. The effectiveness of the therapy was comparable regardless of the period analyzed, and patients achieved an overall response rate of 95%, with an SVR range of 72.9%-100% for the different therapeutic regimens. Male gender, GT3 infection, and prior treatment failure were identified as independent negative predictors of therapeutic success.

Research conclusions

The study documents changes in the profile of HCV-infected cirrhotic patients over the years of accessibility to changing DAA regimens, confirming the high effectiveness of IFN-free therapy in all analyzed periods.

Research perspectives

Changes in the characteristics of patients, especially those with cirrhosis, may affect the expected change in the number of patients with liver cancer or at risk of decompensation. This knowledge is important from the point of view of planning the directions of health care development.

FOOTNOTES

Author contributions: Brzdęk M, Zarębska-Michaluk D, and Flisiak R conceived the study design, and analyzed and interpreted the data; Brzdęk M and Flisiak R prepared the figures; Brzdęk M prepared the tables; Brzdęk M, Zarębska-Michaluk D, and Flisiak R drafted the manuscript; Rzymski P performed the statistical analysis; Lorenc B, Kazek A, Tudrujek-Zdunek M, Janocha-Litwin J, Mazur W, Dybowska D, Berak H, Parfieniuk-Kowerda A, Klapaczyński J, Sitko M, Sobala-Szczygieł B, and Piekarska A acquired the data; Brzdęk M, Zarębska-Michaluk D, Rzymski P, and Flisiak R prepared a revised version of the manuscript; Brzdęk M and Zarębska-Michaluk D prepared the manuscript for the submission; and all authors approved the final version of the manuscript.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Exploring choices of early nutritional support for patients with sepsis based on changes in intestinal microecology

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Abstract

BACKGROUND

Sepsis exacerbates intestinal microecological disorders leading to poor prognosis. Proper modalities of nutritional support can improve nutrition, immunity, and intestinal microecology.

AIM

To identify the optimal modality of early nutritional support for patients with sepsis from the perspective of intestinal microecology.

METHODS

Thirty patients with sepsis admitted to the intensive care unit of the General Hospital of Ningxia Medical University, China, between 2019 and 2021 with indications for nutritional support, were randomly assigned to one of three different modalities of nutritional support for a total of 5 d: Total enteral nutrition

(TEN group), total parenteral nutrition (TPN group), and supplemental parenteral nutrition (SPN group). Blood and stool specimens were collected before and after nutritional support, and changes in gut microbiota, short-chain fatty acids (SCFAs), and immune and nutritional indicators were detected and compared among the three groups.

RESULTS

In comparison with before nutritional support, the three groups after nutritional support presented: (1) Differences in the gut bacteria (Enterococcus increased in the TEN group, Campylobacter decreased in the TPN group, and Dialister decreased in the SPN group; all $P < 0.05$); (2) different trends in SCFAs (the TEN group showed improvement except for Caproic acid, the TPN group showed improvement only for acetic and propionic acid, and the SPN group showed a decreasing trend); (3) significant improvement of the nutritional and immunological indicators in the TEN and SPN groups, while only immunoglobulin G improved in the TPN group (all $P < 0.05$); and (4) a significant correlation was found between the gut bacteria, SCFAs, and nutritional and immunological indicators (all $P < 0.05$).

CONCLUSION

TEN is recommended as the preferred mode of early nutritional support in sepsis based on clinical nutritional and immunological indicators, as well as changes in intestinal microecology.

Key Words: Sepsis; Nutritional support; Intestinal microecology; Short-chain fatty acids; Nutritional and immunological indicators; Total enteral nutrition; Total parenteral nutrition; Supplemental parenteral nutrition

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Core Tip: Nutritional support is an important component of treatment for sepsis, and an appropriate modality of nutritional support can improve patient nutrition, immunity, and intestinal microecology. We applied different nutritional modalities for early and short-duration nutritional support in patients with sepsis and found differences in intestinal bacterial composition, short-chain fatty acids, and nutritional and immune indicators. We concluded that total enteral nutrition is a good modality of early nutritional support for sepsis. These findings provide a new perspective for optimizing nutritional support modalities in sepsis.

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INTRODUCTION

Sepsis is a life-threatening organ dysfunction resulting from a dysregulated response of the body to infection[1]. Sepsis affects nearly 50 million people each year worldwide and accounts for approximately 11 million deaths, corresponding to approximately 20% of all deaths worldwide[2]. The high morbidity and mortality rates associated with sepsis, as well as the difficulties with early detection and treatment, have become challenging healthcare issues.

Our previous study confirmed the presence of gut microbiota disorders in patients with sepsis[3], and the interaction of disease and gut microbiota disorders has been shown to lead to clinical deterioration and the development of multiple organ dysfunction (MODS)[4]. Therefore, the treatment of sepsis requires consideration of the range of its effects on gut microbiota, metabolism, nutrition, and immunity. Nutritional support is an important component of sepsis treatment; however, its focus is still limited to the amount of energy provided, the choice of energy supply routes, the placement of feeding tubes, and nitrogen balance. The clinical recommendations about the choice of nutritional support modalities are largely based on expert consensus, although the supporting evidence is of low level[5,6]. Nutritional support has a significant impact on intestinal microecology; however, the modalities of nutritional support in sepsis have not been evaluated from the perspective of intestinal microecology.

In this context, we used different modalities of nutritional support: Total enteral nutrition (TEN), total parenteral nutrition (TPN), and supplemental parenteral nutrition (SPN) to group patients with sepsis.

Moreover, we explored the choices of nutritional modalities for sepsis from the perspective of systemic immunity and nutritional indicators, the degree of their interrelationships, and the effects of the three nutritional modalities on the intestine microecology [including gut microbiota, short-chain fatty acid (SCFA) metabolism]. In this study, 16S rRNA gene sequencing and GC-MS metabolomics were used to detect gut microbiota and SCFAs. The exploration of early nutritional support in patients with sepsis from the perspective of intestinal microecology not only provides a new fundamental perspective for optimizing nutritional support in sepsis but also has far-reaching implications for improving nutritional support.

MATERIALS AND METHODS

General information

A randomized clinical trial involving 30 patients with sepsis admitted to the Department of Critical Care Medicine at the General Hospital of Ningxia Medical University (a Grade 3A hospital) between October 1, 2019, and February 1, 2021, was conducted. The inclusion criteria were as follows: (1) Age between 18 and 75 years; (2) meeting the criteria of the latest definition of sepsis of the American Society of Critical Care Medicine "Sepsis-3.0" from 2016[1]; (3) mechanically ventilated patients; and (4) indications for nutritional support [hemodynamic stability (no dose or small dose of vasoactive drugs to maintain vital signs and lactate ≤ 2 mmol/L) and NUTRIC score (Nutritional Risk Rating Instrument for Critically Ill Patients) ≥ 6]. The following exclusion criteria applied: (1) Patients with chest wall, thoracic, or airway injuries, respiratory instability, ventilator parameters requiring adjustment during measurement, or rapid respiratory rate ≥ 35 breaths/min, or oxygen concentration > 60%, or positive end-expiratory pressure ≥ 10 cmH_2O; (2) patients with open injuries to the gastrointestinal tract or abdominal cavity; (3) patients with severe acid-base balance or electrolyte metabolism disturbances; and (4) patients whose family members did not provide informed consent to participate in the trial. Informed consent was obtained from the patients or their immediate family members. This study was approved by the Ethics Committee of the Hospital (approval number: 2017-266).

Patients with sepsis included in this study were divided into three groups using a random number table and a random number remainder grouping method. Nutritional support was administered to the patients *via* the following different modalities based on the order of randomization: (1) TEN group (n = 10): Total enteral nutritional support *via* a gastric tube or jejunal tube feeding; (2) TPN group (n = 10): Total parenteral nutrition *via* the internal jugular vein; and (3) SPN group (n = 10): Trophic enteral nutrition *via* a gastric tube or jejunal tube feeding + SPN *via* the internal jugular vein, in which trophic enteral is 10–20 kcal/h, not more than 500 kcal in 24 h, and the remaining energy is supplemented by parenteral nutrition[6]. The amount of nutritional support provided to the patients in the three groups was based on the resting energy expenditure (REE) measured by indirect calorimetry in the ventilation circuit; the energy metabolism test (metabolic cart) used in this study was Vmax Encore 229, Medical Graphics, United States, 2015. The selected SPN enteral preparation for enteral nutrition solution was Enteral Nutritional Suspension (SP, 500 mL/500 kcal, Nutricia Pharmaceutical Co., Ltd.). The selected TEN enteral preparation for enteral nutrition solution, due to energy supply and fluid volume regulation, was Enteral Nutritional Emulsion (TPF, total energy 500 mL/750 kcal, Nutricia Pharmaceutical Co., Ltd.), combined or not with SP. Patients who received nutritional support for less than 5 d during the nutritional support period because of changes in patient condition (discharge from hospital after improvement in condition or inability to continue with nutritional support owing to deterioration in condition) were regarded as invalid observation cases and excluded from the study. Subsequently, the next randomized patient was administered nutritional support using the same modality used for the invalid case. Patient age, sex, underlying disease, NUTRIC score, Acute Physiology and Chronic Health Evaluation (APACHE II) score, sequential organ failure assessment (SOFA) score, and general information such as REE during 24 h, were recorded. Before day 1 and on day 5 of nutritional support, stool specimens were collected for 16S rRNA gene sequencing and the analysis of SCFA metabolism and venous blood specimens were collected for lymphocyte subpopulation analysis, immunoglobulin + complement analysis, and complete set of nutritional tests.

Fecal specimen collection and sequencing

Stool specimens were collected from enrolled patients with sepsis on days 1 and 5 of nutritional support. Specimens were collected from the bottom layers of fresh stools of the patients with a sampling spatula after spontaneous defecation or a warm saline enema. The stool specimens were quickly placed in stool specimen boxes, which were enveloped, labeled, and stored in liquid nitrogen tanks and transferred to a -80 °C refrigerator for freezing.

Each stool sample was spiked with 790 μL of lysis solution [4M guanidine thiocyanate, 250 μL; 10% N-lauroyl sarcosine, 40 μL; 5% N-lauroyl sarcosine-0.1 M phosphate buffer (pH 8.0), 500 μL], followed by a vigorous vortex mixing, and then incubated at 70 °C for 1 h. At the end of the incubation, bead-beating with glass beads (0.1 mm, 500–750 μL) was performed for 10 min (25HZ/S); subsequent extraction was performed according to the instructions of the extraction kit (E.Z.N.A.® Stool DNA Kit).

The concentration of extracted DNA was measured using NanoDrop (Thermo Scientific). The V3-V4 region of the 16S rRNA gene was amplified by primers 341F/805R (341F: *5'-CCTACGGGNGGCWGCAG-3'*; 805R: *5'-GACTACHVGGGTATCTAATCC-3'*), and the PCR products were sequenced using an Illumina Miseq 2 × 300 bp platform.

Short-chain fatty acid metabolism test

Standard configuration: Appropriate amounts of pure acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and caproic acid were used to prepare 10 mixed concentration gradients (0.02 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 2 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, and 500 μg/mL). The master mixes and working standard solutions were stored at 0 °C.

Sample pre-treatment: Appropriate amount of sample was added along with 50 μL of 15% phosphoric acid, 100 μL of 125 μg/mL of internal standard (isocaproic acid), and 400 μL of ether. The solution was then homogenized for 1 min, centrifuged at 4 °C for 10 min at 12000 rpm, and the supernatant was collected for testing.

GC-MS detection method: The chromatographic conditions were as follows: Agilent HP-INNOWAX capillary column (30 m × 0.25 mm ID × 0.25 μm); split injection, injection volume 1 μL, split ratio 10 : 1; inlet temperature 250 °C; ion source temperature (eng) 230 °C; transmission line temperature 250 °C, quadrupole temperature 150 °C. The temperature ramp-up time was programmed to start at 90 °C, ramping up to 120 °C at 10 °C/min, to 150 °C at 5 °C/min, and ultimately reaching 250 °C at 25 °C/min for 2 min. The carrier gas was helium at a flow rate of 1.0 mL/min.

Blood test methods

Lymphocyte subpopulation analysis: Flow cytometry method, contained T lymphocytes (CD3+), B lymphocytes (CD3- CD19+), and NK lymphocytes (natural killer cells, CD3- CD16+ and/or CD56+); T cells were subdivided into helper T cells (CD3+ CD4+) and suppressor T cells (CD3+ CD8+). The DxFLEX flow cytometry (lymphocyte division) analyzer (Beckman Coulter Ltd., United States) and lymphocyte assay kit (Beckman Coulter Ltd., United States) were used.

Immunoglobulin + complement, nutritional panel (scattering and turbidimetric method): The nutritional panel contained albumin (ALB), prealbumin (PAB), and transferrin; immunoglobulin and the complement tests contained immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), C3, and C4. BN II analyzer (SIEMENS GmbH, Germany). The IgM assay kit as well as the nutritional complete assay kit (SIEMENS Healthcare Diagnostics GmbH, Germany) were used.

Statistical methods

Clinical data were processed and analyzed using the Statistical Package for Social Sciences version 19.0 (IBM, Armonk, New York), and the data were first tested for normality and variance. Normally distributed variables and variance measures were expressed as mean ± SD, using *t*-test and analysis of variance; non-normally distributed measures were expressed as M (P25, P75), using a rank sum test; χ^2 test was used for the comparison of frequency data. A P value < 0.05 was considered statistically significant.

Raw sequencing data were processed using USEARCH (version 11.0.667) and operational taxonomic units (OTUs) were classified based on 97% sequence similarity, and representative OTU sequences were compared with the SILVA database (SSU138, http://www.arb-silva.de) to obtain the taxonomic classification of each 16S rDNA sequence. The α-diversity of each sample was assessed using Ace, Chao 1, Shannon-Wiener diversity index, and Simpson diversity index, and differences between the groups were tested using the non-parametric Mann–Whitney U test or Kruskal–Wallis rank sum test. Principal coordinates analysis and Linear discriminant analysis Effect Size analysis were performed and heat maps were constructed using R statistical software. Spearman correlation analysis was performed on gut microbiota, SCFA metabolism, and clinical nutritional and immunological indicators of the patients with sepsis treated with different modalities of nutritional support for 5 d.

RESULTS

Characteristics of the participants

Patients with sepsis were grouped into TEN, TPN, and SPN groups according to different nutritional modalities, and basic patient data were compared (Table 1). There were no significant differences between the groups in terms of sex, age, underlying disease, and body mass index (P > 0.05). The APACHE II and SOFA scores were compared among the three groups, and no significant differences were observed in the degree of severity of sepsis (P > 0.05). Furthermore, the NUTRIC score was ≥ 6 in all three groups, and no significant differences were observed (P > 0.05). The daily REE of patients with

Table 1 Comparison of the general information among the three groups of individuals

Indicator	TEN (*n* = 10)	TPN (*n* = 10)	SPN (*n* = 10)	H/F/χ^2/Z/*t* value	*P* value
Age (yr, mean ± SD)	59.20 ± 12.52	54.30 ± 15.10	59.30 ± 16.33	0.46	0.63
Sex					
Male (case, %)	8 (80)	8 (80)	8 (80)	0.00	1.00
Female (case, %)	2 (20)	2 (20)	2 (20)		
Underlying disease					
Hypertension (case, %)	4 (40)	3 (30)	5 (50)	0.83	0.65
Diabetes (case, %)	2 (20)	4 (40)	2 (20)	1.36	0.50
Malignant tumor (case, %)	0 (0)	2 (20)	2 (20)	2.30	0.31
APACHE II score on day 1 (points, mean ± SD)	14.10 ± 2.84	15.30 ± 4.73	19.50 ± 7.38	2.12	0.14
SOFA score on day 1 (points, mean ± SD)	5.40 ± 1.51	9.60 ± 1.95	10.20 ± 4.10	5.53	0.10
BMI (kg/m^2, mean ± SD)	23.90 ± 2.79	26.84 ± 3.89	26.05 ± 2.67	1.90	0.17
NUTRIC score on day 1					
≥ 6 points (case, %)	3 (30)	6 (60)	6 (60)	2.40	0.30
< 6 points (case, %)	7 (70)	4 (40)	4 (40)		
REE on day 1 [kcal, M (P25, P75)]	1950.00 (1759.28, 2050.72)	1900 (1726.40, 2073.59)	2036 (1882.46, 2189.53)	2.49	0.28

APACHE II score: Acute Physiologic Assessment and Chronic Health Evaluation II Scoring System; SOFA score: Sequential Organ Failure Assessment Score; BMI: Body mass index; NUTRIC score: Nutritional Risk Rating Instrument for Critically Ill Patients; REE: Resting energy expenditure; TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition.

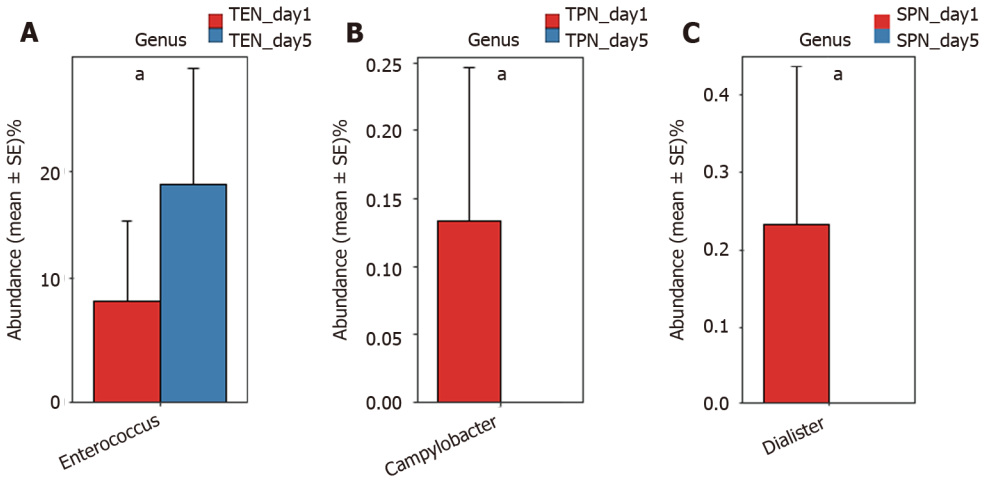
sepsis obtained by indirect calorimetry performed by the ventilation circuit was used as the set amount for nutritional support, and no significant difference was observed among the three groups in the 24-h REE ($P > 0.05$). General information were balanced and comparable between the groups.

Effect of different nutritional modalities on gut microbiota in patients with sepsis

No significant improvement in α-diversity and β-diversity of gut microbiota was observed in the three groups, before and after nutritional support (Supplementary Figures 1 and 2) ($P > 0.05$).

Comparison of gut microbiota composition at the genus and OTU levels: The Mann-Whitney U test was separately performed in each group to compare the genera of the gut microbiota before and after nutritional support. Results indicated that compared with the gut microbiota of patients with sepsis before nutritional support, significant changes occurred in some genera of the gut microbiota after 5 d of nutritional support. Specifically, Enterococcus was significantly higher in the TEN group after nutritional support ($P < 0.05$) (Figure 1A); Campylobacter was significantly lower in the TPN group after nutritional support ($P < 0.05$) (Figure 1B); and Dialister was significantly lower in the SPN group after nutritional support ($P < 0.05$) (Figure 1C).

A random forest approach was used to compare the OTU level of the three groups, and changes in key bacterial OTUs before and after nutritional support were identified. After nutritional support in the TEN group, we obtained a significant reduction in the relative abundance of 10 OTUs, including those belonging to the genera Streptococcus, Methylobacterium-Methylorubrum, Oscillospiraceae *UCG-005*, Faecalibacterium, Escherichia-Shigella, Eubacterium coprostanoligenes group, Agathobacter, and Hungatella. Furthermore, the relative abundance of one OTU belonging to the genus Enterococcus and one unclassified OTU belonging to the Enterobacteriaceae family significantly increased (Figure 2A). After nutritional support in the TPN group, we found a significant reduction in the relative abundance of 16 OTUs, including those belonging to the genera Anaerococcus, Prevotella, Porphyromonas, Methylobacterium-Methylorubrum (2 OTUs), Faecalibacterium (2 OTUs), Ochrobactrum, Sphingomonas, Phyllobacterium, Agathobacter, Blautia, Pelomonas, and Acidovorax, while the relative abundance of the two OTUs belonging to the genus Enterococcus significantly increased (Figure 2B). After nutritional support in the SPN group, we found a significant reduction in the relative abundance of 11 OTUs, belonging to the genera Dialister, Serratia, Streptococcus, Porphyromonas, Anaerostipes, Veillonella, Fusicatenibacter, Escherichia-Shigella, and Ezakiella, and two unclassified OTUs in the families Lachnospiraceae and Ruminococcaceae (Figure 2C).



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Figure 1 The key bacterial taxa significantly changed before and after nutrition support at the genus level. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Total enteral nutrition; B: Total parenteral nutrition; C: Supplemental parenteral nutrition. The relative abundance of each genus is depicted as the mean ± SE. *P* values were calculated using the Mann–Whitney U test: [a]*P* < 0.05. TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition.

Effect of different nutritional modalities on short-chain fatty acid metabolism in patients with sepsis

The GC-MS assay was used to detect fecal SCFAs before and after nutritional support in the three groups. The changes in the SCFAs before and after nutrition in the TEN (Figure 3), TPN (Figure 4), and SPN (Figure 5) groups were compared and all SCFAs in the TEN group showed an increasing trend except for caproic acid. Acetic and propionic acid in the TPN group showed an increasing trend, while all SCFAs in the SPN group showed a decreasing trend. However, no significant difference ($P > 0.05$) was observed in the changes of SCFAs by nutritional support pathways in patients with sepsis, both before and after group nutrition and between groups.

Effect of different nutritional modalities on immune and nutritional indicators in patients with sepsis

Paired sample *t*-test and non-parametric test for correlated samples were used to compare the immune and nutritional indicators of the three groups before and after nutrition. The results of these analyses are shown in Table 2. After nutritional support in the TEN group, CD3+ T cells, CD3+/CD4+ T cells, IgG, complement C3, PAB, and ALB levels significantly increased, while CD3- CD19+ B cells significantly decreased ($P \leq 0.05$). In the TPN group, only the IgG level significantly increased after nutritional support ($P = 0.007$), while the remaining immune and nutritional indicators did not change significantly ($P > 0.05$). In the SPN group, IgA, IgG, complement C3, PAB, ALB, and transferrin levels significantly increased, while CD3- CD19+ B cell levels significantly decreased after nutritional support ($P \leq 0.05$).

To avoid the influence of immune and nutritional indicators of patients in each group before nutritional support on indicators after nutrition, analysis of covariance was used to compare the three groups. Post-nutritional pre-ALB was significantly higher in the TEN group than in the TPN group ($P = 0.05$), while ALB was significantly higher in the SPN group than in the TPN group ($P = 0.019$).

Correlation analysis between gut bacteria abundance and SCFAs metabolism as well as nutritional immune indicators after nutritional support for patients with sepsis

Spearman correlation analysis revealed that after nutritional support there was a correlation between the more dominant species in the gut and SCFAs and clinical immune and nutritional indicators in patients with sepsis (Figure 6). For example, Enterococcus spp., which increased in abundance in the TEN group after the nutritional intervention, showed a significant positive correlation with IgA; Streptococcus and Escherichia-Shigella spp., which decreased in abundance, showed a significant negative correlation with PAB concentrations, as well as a significant positive correlation with intestinal concentrations of multiple SCFAs. Methylobacterium spp. were significantly negatively correlated with IgG and SCFA concentrations (all $P < 0.05$). Anaerococcus and Porphyromonas spp., which decreased in the TPN group, showed a significant positive correlation with PAB concentrations, and Methylobacterium, Sphingomonas, and Phyllobacterium spp. showed a significant negative correlation with IgG and multiple SCFAs concentrations (all $P < 0.05$). Streptococcus and Escherichia-Shigella, which showed decreased abundance in the SPN group, were negatively correlated with PAB concentrations and also positively correlated with multiple SCFA concentrations; Porphyromonas and Anaerostipes were positively correlated with PAB concentrations (all $P < 0.05$). In addition, no significant correlations were observed between clinical nutritional immune indicators such as ALB, CD3+ T cells, CD3- CD19+ B

Table 2 Effect of different nutritional modalities on immune and nutritional indicators in patients with sepsis

Indicator	TEN (*n* = 20)				SPN (*n* = 20)				TPN (*n* = 20)			
	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value
CD3+ T cell [%, M (P25, P75)]	69.85 (63.62, 78.77)	77.77 (69.40, 80.48)	2.09	0.03	71.33 (64.42, 76.50)	72.25 (64.59, 82.59)	1.78	0.07	74.21 (59.82, 83.43)	74.80 (67.23, 82.74)	0.76	0.44
CD3+ CD4+ T cell [%, M (P25, P75)]	36.40 (29.47, 43.39)	40.39 (36.96, 46.50)	1.98	0.04	38.21 (32.22, 57.15)	47.37 (35.61, 58.27)	1.17	0.24	38.12 (29.23, 46.40)	45.67 (28.13, 51.69)	1.37	0.16
CD19+ B cell [%, M (P25, P75)]	17.04 (11.55, 21.27)	14.11 (8.99, 18.03)	1.98	0.04	16.80 (10.70, 25.14)	9.75 (6.47, 16.59)	2.09	0.03	14.35 (6.62, 22.89)	12.78 (6.93, 23.61)	1.02	0.30
IgA (g/L mean ± SD)	2.53 ± 1.18	2.93 ± 0.93	1.97	0.08	2.15 ± 0.83	2.91 ± 1.19	3.87	0.00	2.10 ± 1.13	2.45 ± 0.73	1.44	0.18
IgG (g/L, mean ± SD)	11.75 ± 3.46	15.19 ± 5.84	3.10	0.01	9.05 ± 3.78	13.77 ± 4.52	5.48	0.00	9.32 ± 3.89	12.15 ± 3.85	3.48	0.00
IgM (g/L, mean ± SD)	0.81 ± 0.36	1.11 ± 0.48	1.49	0.16	0.95 ± 0.44	1.12 ± 0.75	1.11	0.29	0.77 ± 3.89	0.89 ± 0.46	1.02	0.33
C3 (g/L, mean ± SD)	0.99 ± 0.30	1.21 ± 0.23	2.84	0.01	0.81 ± 0.21	1.07 ± 0.23	4.73	0.00	0.77 ± 0.25	0.88 ± 0.33	0.95	0.36
C4 (g/L, mean ± SD)	0.19 ± 0.04	0.22 ± 0.05	2.08	0.06	0.19 ± 0.07	0.22 ± 0.07	3.64	0.00	0.17 ± 0.05	0.21 ± 0.08	1.75	0.11
Albumin (g/L, mean ± SD)	27.14 ± 3.18	29.89 ± 3.61	3.04	0.01	27.74 ± 3.45	30.97 ± 2.63[a]	2.68	0.02	28.10 ± 3.37	27.73 ± 2.64	0.24	0.81
Pre-albumin (g/L, mean ± SD)	0.10 ± 0.04	0.15 ± 0.07[a]	3.88	0.00	0.08 ± 0.41	0.13 ± 0.05	3.42	0.00	0.10 ± 0.03	0.11 ± 0.03	0.47	0.64
Transferrin (g/L, mean ± SD)	0.01 ± 0.28	0.01 ± 0.28	1.52	0.15	1.16 ± 0.36	1.36 ± 0.51	2.67	0.02	1.05 ± 0.30	1.20 ± 0.37	1.11	0.29

[a]$P \leq 0.05$ compared with the enteral nutrition group.

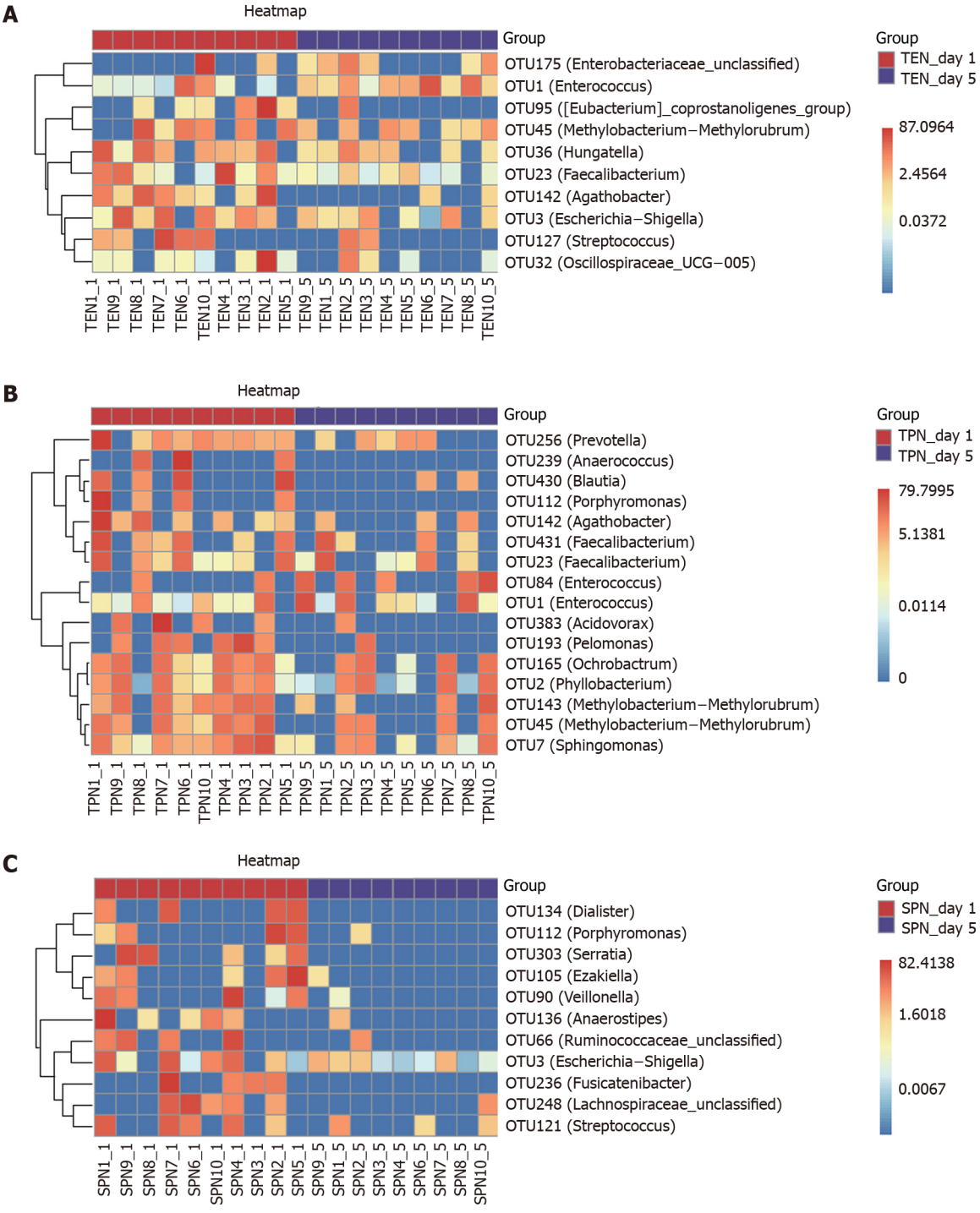
TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition; day 1: Before nutrition support; day 5: After nutrition support; IgA: Immunoglobulin A; IgM: Immunoglobulin M; IgG: Immunoglobulin G.

cells, gut bacteria, and SCFAs (all $P > 0.05$).

DISCUSSION

Effect of different nutritional modalities on gut microbiota diversity and species composition in patients with sepsis

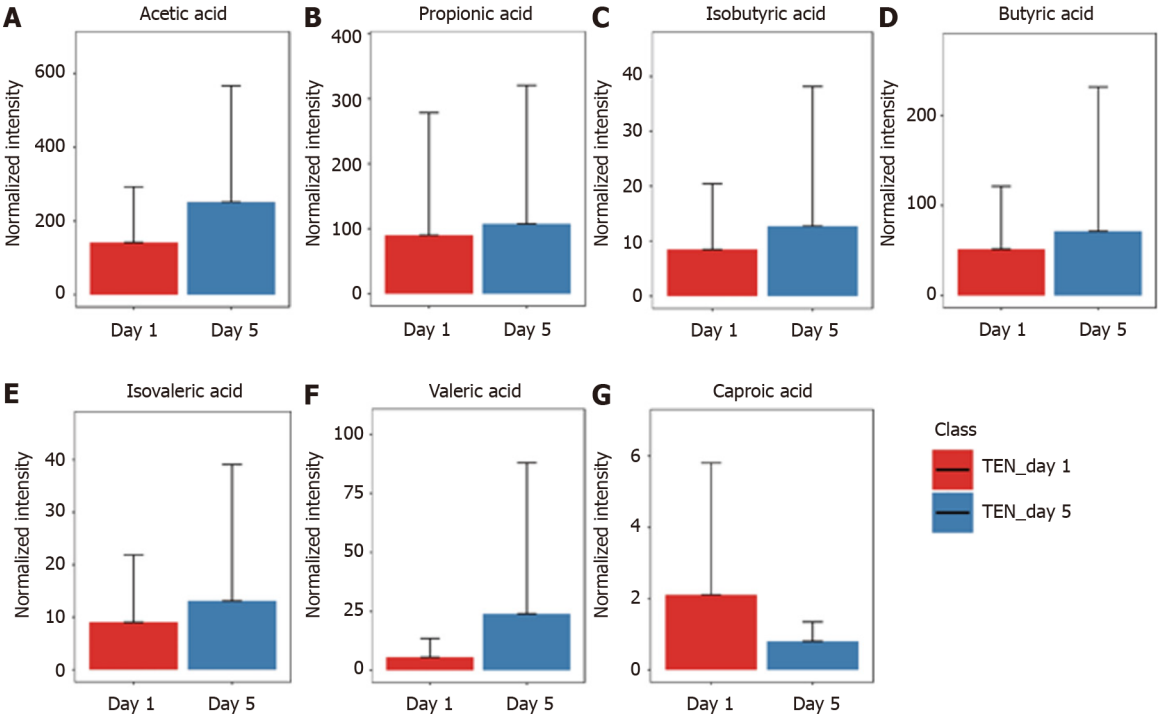
By comparing the gut microbiota of septic patients, non-septic patients, and healthy controls, our previous study confirmed that septic patients undergo gut microbiota disorders, which persist for a week or longer[3]. This is consistent with the current knowledge of the characteristics of gut microbiota disorders in patients with sepsis[7,8]. Gut microbiota disorders contribute to the development of sepsis through the proliferation of pathogenic bacteria, dysregulated immune response, and reduced production of microbiota-derived metabolites, such as SCFAs. After the onset of sepsis, alterations in the structure of the normal gut microbiota can exacerbate the condition, leading to a worsening of the prognosis for sepsis, and the development of MODS[4].



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Figure 2 Heatmaps display the key gut microbial operational taxonomic units influenced by three types of nutritional support. A: Total enteral nutrition; B: Total parenteral nutrition; C: Supplemental parenteral nutrition. The key operational taxonomic units are selected by Random Forest approach according to the changes of relative abundance before and after nutritional support. TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition; OTU: Operational taxonomic unit.

Nutritional support is an important part of the treatment of critically ill patients. Diet is by far the most significant among the factors known to affect the gut microbiota[9,10]. Studies have found that diet under normal physiological conditions can affect the gut flora in the short-term, rapidly, and significantly[11]. In this study, we found that nutritional support for 5 d using different modalities of nutritional support did not show significant improvement in the gut microbiota disorders of patients with sepsis. This is in line with the perception of gut microbiota disorders in sepsis. Although the mechanism of intestinal microecological disorders due to sepsis is not clear, multiple factors such as antibiotics[12], sedative and analgesic drugs[13], nutritional support[14], and proton pump inhibitors [15] affect gut flora among patients with sepsis, making it impossible for a single factor to significantly



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Figure 3 The changes of short-chain fatty acids before and after nutrition support in the total enteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. TEN: Total enteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD.

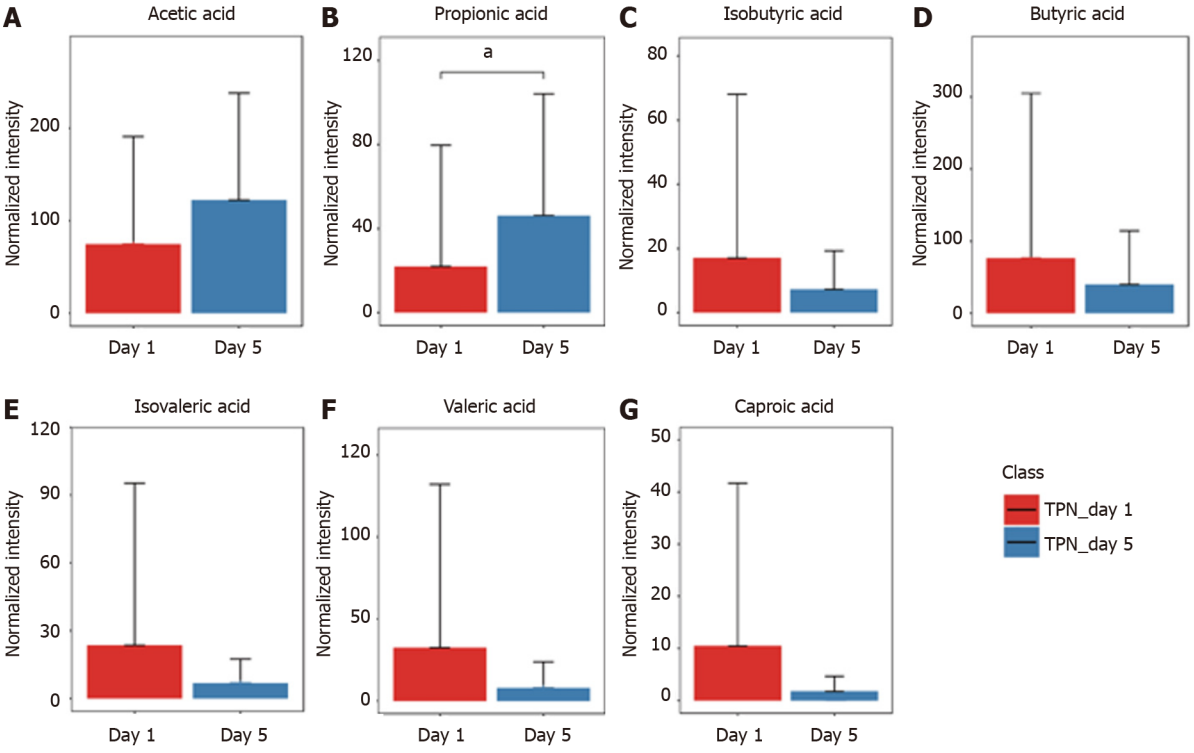
improve the condition in a short period of time.

Although short-term nutritional support could not correct gut microbiota disorders in patients with sepsis, different nutritional support modalities caused some differences in bacterial species: Enterococcus spp. were significantly high in the TEN group, Campylobacter spp. were significantly low in the TPN group, and Dialister spp. were significantly low in the SPN group. In animal studies, it has been demonstrated that Enterococcus can effectively improve animal growth[16,17], enhance the absorption of other nutrients[18], and is a beneficial additive, which is widely used in animal feeds. Current research suggests that drug-resistant strains of Enterococcus may be pathogenic[19]; however, its commensal strains have been identified as probiotics in animal and human intestines[20]. Further studies have shown that Enterococcus can trigger immune signaling pathways and regulate infection [21], autoimmunity[22]. Therefore, we believe that a significant increase of Enterococcus spp. after TEN treatment suggests that early TEN support can improve gut microbiota in sepsis. Campylobacter spp. is the main cause of gastroenteritis in humans, and consumption of contaminated poultry meat is its main transmission route. The significant decrease of this genus after TPN treatment suggests improvements in gut microbiota in sepsis; however, the underlying mechanism is currently unclear and requires further study. Non-oral ingestion during TPN support may have cut-off the transmission route of Campylobacter spp. Other reports suggest that a variety of probiotic bacteria in the intestine reduce the severity of Campylobacter spp. infection by negatively affecting the virulence and survival factors (*e.g.*, adhesion, invasion) of various Campylobacter spp., attenuating intestinal inflammation[23]. Dialister spp. may be positively associated with glucose metabolism disorders, obesity, and insulin resistance[24, 25], and our previous study found that Dialister spp. in patients with sepsis was positively associated with diamine oxidase, a highly active intracellular enzyme in the upper villi of the small intestinal mucosa in humans and mammals. Elevated diamine oxidase reflects the degree of damage to the intestinal mechanical barrier[4]. Therefore, the significant decrease in Dialisteria spp. suggests that SPN may improve intestinal barrier function.

Early, short-term nutritional support in sepsis has a beneficial effect on gut bacteria composition, although it did not completely improve gut microbiota disorders.

Effects of different nutritional modalities on SCFAs in patients with sepsis

SCFAs are the main metabolites produced by specific symbiotic bacteria of the gut after fermentation of dietary fiber and indigestible polysaccharides and starches, including acetic, propionic, butyric, isobutyric, pentanoic, isovaleric, and caproic acid. Among them, acetic, propionic, butyric, and isovaleric acid are the most common SCFAs. In addition to their ability to provide direct nutrition to intestinal commensal microorganisms, SCFAs positively influence host immune cell differentiation,



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Figure 4 The changes of short-chain fatty acids before and after nutrition support in the total parenteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. TPN: Total parenteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD. *P* values were calculated using the Student's *t*-test: $^{a}P < 0.05$.

immune system, and metabolism as well as modulate host susceptibility[26,27]. Furthermore, they have a protective effect on intestinal barrier function[28,29].

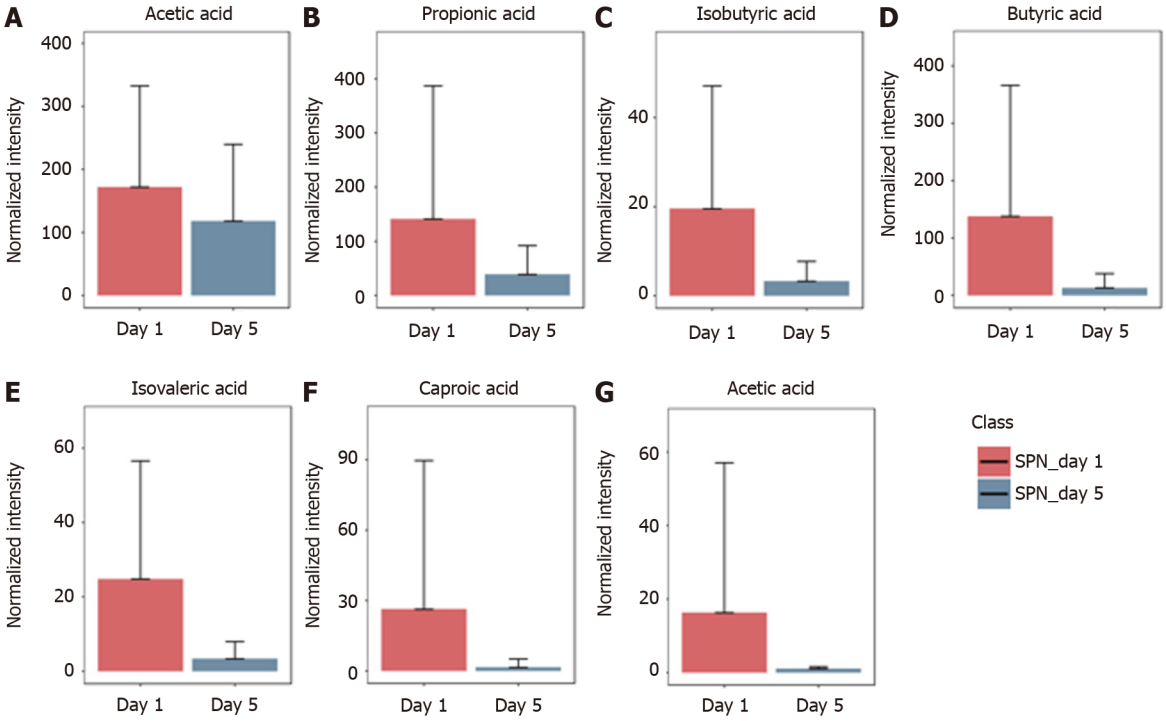
In this study, we found that there was no significant improvement in SCFAs after 5 d of nutritional support in patients with sepsis. This is complemented by the fact that gut microbiota disorders in sepsis are not effectively corrected, and a close correlation exists between SCFAs and gut microbiota, and SCFAs cannot be significantly improved in the absence of effective correction of gut microbiota disorders. In patients with sepsis, there is a significant decrease in SCFA-producing bacteria in the intestine, such as Bifidobacterium and Bacillus. This can cause a dramatic decrease in SCFAs and cause intestinal barrier dysfunction. Concomitantly, decreased levels of SCFAs can further aggravate the gut microbiota disorders in sepsis[30,31].

In this study, we found that all SCFAs in the TEN group showed an increase trend except for caproic acid. In the TPN group, acetic acid showed an improving trend and propionic acid improved significantly, while the remaining SCFAs did not show any improvement. SCFAs in the SPN group showed a continuous decreasing trend. The complete bypassing of the intestine by TPN can serve as both advantage and disadvantage[32]. TPN lacks key intestinal luminal nutrients compared to TEN, resulting in a significant deficiency of glutamine and SCFAs[33,34]. Animal studies have shown that this deficiency cannot be effectively improved by the addition of glutamine and SCFAs to intravenous nutrition[34-36]. Supplemental enteral nutrition during SPN mostly uses ready-made enteral preparations with low fiber or without fiber, which not only fails to promote the production of SCFAs but can also form pathogenic microorganisms with higher virulence[30,37]. The reason for this may be the inability of highly absorbable trophic preparations to adequately feed the intestinal epithelium and intestinal commensal bacteria, leading to disruption of the intestinal barrier, stress signaling, and proliferation of intestinal pathogenic bacteria[38].

Although nutritional support in early sepsis did not significantly improve SCFA metabolism, different nutritional support modalities had different effects on SCFAs. Moreover, TEN had a tendency to improve overall SCFA production in sepsis compared to TPN and SPN and was theoretically more conducive to SCFA production due to dietary fiber supplementation and adequate enteral nutritional supplementation.

Effects of different nutritional modalities on nutritional and immunological indicators in patients with sepsis

In this study, we found that in early and short-term nutritional support for patients with sepsis, TEN

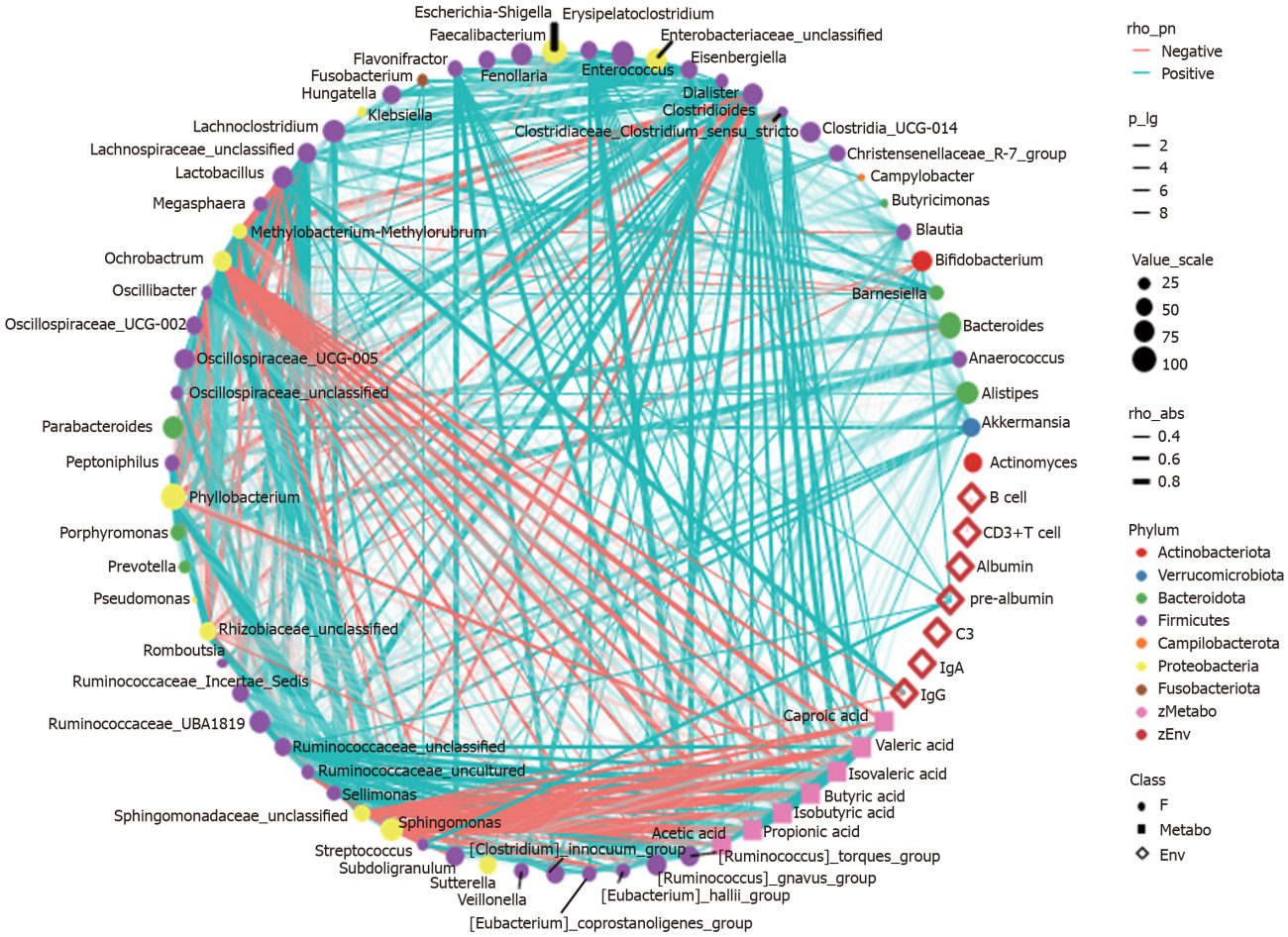


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Figure 5 The changes of short-chain fatty acids before and after nutrition support in the supplemental parenteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. SPN: Supplemental parenteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD.

can significantly improve cellular immunity, humoral immunity, and nutrition-related indicators; SPN can significantly improve humoral immunity and nutritional indicators; and TPN can only improve IgG. The effects of the disease, vascular leakage, fluid supplementation, diuresis, bed confinement, and other factors, make weight assessment difficult for patients with sepsis. Additionally, muscle mass and other assessment indicators do not truly reflect the nutritional changes of the patient. Therefore, it is more appropriate to select plasma proteins as an indicator of nutrition in patients with sepsis. These proteins also need to have relatively small storage, rapid synthesis, and stable metabolic rate. Pre-ALB has a half-life of only 48 h, a small body storage pool, responds to negative nitrogen balance in the body in a short time, and is a considerably more sensitive indicator of malnutrition than either ALB or transferrin. Combined with the effect on PAB in the three groups in this study, early nutritional support of TEN in sepsis significantly improved the nutritional status of patients compared with TPN. Interestingly, PAB concentration was negatively correlated with the relative abundance of OTUs of Streptococcus and Escherichia-Shigella, while it was positively correlated with the relative abundance of OTUs of Anaerococcus and Porphyromonas. Additionally, the corresponding species were significantly lower in TEN and TPN, respectively. Further studies are needed to clarify whether this may suggest a relationship between improved nutrition and gut microbiota.

The intestine is the largest immune organ in the body. Studies in mice have shown that nutritional pathways and typology can affect gut-associated lymphoid tissue[39]. Relative to TPN, TEN reduces the occurrence of potential gastrointestinal complications, including maintenance of the intestinal epithelial barrier and tight junctions, increases constituent proteins, reduces acute phase reactants and bacterial translocation, and enhances mucosal immunity[40]. A meta-analysis showed that enteral feeding and sepsis are negatively correlated[41]. A more plausible explanation at present is that TPN lacks intraluminal nutrients, such as glutamine and SCFAs, which are essential for local enterocytes and colon cells, and that delivery of these nutrients *via* extra-gastrointestinal routes does not directly deliver nutrients to the most nutrient-demanding cells of the gastrointestinal tract. In contrast, key intestinal-systemic signaling regulators were significantly downregulated in animals fed *via* TPN[34]. These findings explain why TEN and SPN in this study could improve nutritional and immune indicators early in patients with sepsis, while TPN did not significantly improve these indicators. Related studies have also shown that, in addition to the nutritional pathway, the composition and adequacy of nutrients in the intestinal lumen determine the gene expression of host intestinal receptors as well as the uptake of luminal metabolites and hormonal signaling[36]. Approximately 20% of the energy supply of SPN passes through the gastrointestinal tract, which allows early improvement of nutrition and humoral immunity in patients with sepsis; however, due to the single formulation of the provided preparation, the frequent absence of dietary fiber, and the insufficient total energy supply through the intestine, SPN



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Figure 6 Correlation analysis between gut microbiomes, short-chain fatty acids and nutritional immune indicators after nutritional support for patients with sepsis. Network graph constructs based on Spearman's correlation analysis. The results showed correlations among 60 predominant fecal microbial genera, 7 short-chain fatty acids (SCFAs) and 7 clinical immune and nutritional indicators. Connecting lines represent the significant correlation ($P < 0.05$). Red lines indicate negative correlations, blue lines indicate positive correlations, and the width of the lines represents the strength of the correlation. The transparency of the lines represented the negative logarithm of the *P* value of correlation. The size of the points indicates the relative abundance of genera. The colors of points display the different phyla of the microbiome. The circle represents the fecal microbiome, the square represents the SCFAs, and the diamond represents plasma metabolites.

does not improve cellular immunity and SCFAs during early nutritional support in sepsis.

IgG and IgA are important immunoglobulins that interact with the gut microbiota to exert corresponding immune effects[42]. In this study, the intestinal Enterococcus spp. and IgA were positively correlated and Methylobacterium spp. and IgG were negatively correlated in patients with sepsis. Accordingly, these two species also showed significant increase and decrease in the TEN group, which suggested interaction between IgG, IgA, and gut microbiota. Meanwhile, the correlation between Enterococcus spp. and IgA may be one of the mechanisms by which Enterococcus spp. exert beneficial effects on the intestinal microecology in sepsis, which remains to be further investigated.

The present study is a preliminary study investigating the modalities of early nutritional support in patients with sepsis from the perspective of intestinal microecology. However, this study is limited by the small sample size and short duration of nutritional support; therefore, significant improvements in intestinal microecology did not have time to materialize. The only differences are observed in the bacterial genera and changes in SCFAs. However, the available preliminary results provide an insight into the effects of different modalities of nutritional support on intestinal microecology, and their link to host nutrition and immunity, suggesting that TEN may be a more appropriate approach for early nutritional support in patients with sepsis. How different nutritional support modalities affect the structural and metabolic changes of gut microbiota composition and the related mechanisms involved need to be studied and explored by designing clinical trials with larger scale and longer intervention time, which is the next direction of our research.

CONCLUSION

In this study, three groups were classified on the basis of different modalities of nutritional support for sepsis. By comparing the changes in gut microbiota structure, species composition, and SCFAs, early, short-term nutritional support could not completely improve intestinal microecological disorders in sepsis, and accordingly, there was no significant improvement in SCFAs. However, the differences in the species composition of intestinal bacteria suggested a beneficial effect of early nutritional support on gut microbiota disorders. Relative changes in SCFAs showed that TEN support had an improving trend on the indicators. The changes in clinical nutritional and immune indicators before and after nutritional support showed that early TEN support largely improved cellular immunity, humoral immunity, and nutritional indicators. Moreover, TEN support improved clinical nutritional indicators of patients better than TPN. Meanwhile, the correlation between gut microbiota and SCFAs and clinical nutritional and immune indicators suggested some level of interaction. In conclusion, the results of this study suggest that TEN should be the recommended modality for early nutritional support in sepsis from the perspective of intestinal microecology. These findings offer a novel perspective for optimizing the mode of nutritional support in sepsis.

ARTICLE HIGHLIGHTS

Research background

Sepsis is a common disease in intensive care units, with high morbidity and mortality. Our previous study confirmed the presence of gut microbiota disorders in patients with sepsis. The combination of disease and gut microbiota disorders lead to the development of multiple organ dysfunction and clinical deterioration of the patient. Nutritional support is an important part of the treatment of critically ill patients. Proper modalities of nutritional support can improve nutrition, immunity, and intestinal microecology. The exploration of early nutritional support in patients with sepsis from the perspective of intestinal microecology is important to optimize nutritional support and improve prognosis.

Research motivation

The recommendations about the choice of nutritional support modalities are largely based on expert consensus, although the level of evidence is low. Nutritional support has a significant impact on intestinal microecology; to date, the modalities of nutritional support in sepsis have not been evaluated from the perspective of intestinal microecology. This perspective can provide new insights into the optimization of the modalities for nutritional support in sepsis.

Research objectives

The main objective was to determine the optimal modality of early nutritional support for patients with sepsis from the perspective of intestinal microecology. We applied different nutritional modalities for early and short-duration nutritional support in patients with sepsis and found differences in intestinal bacterial composition, short-chain fatty acids (SCFAs), and nutritional and immune indicators. Our results revealed for the first time that total enteral nutrition (TEN) is a good modality for early nutritional support in patients with sepsis. This study offers a new perspective for optimizing nutritional support modalities in sepsis.

Research methods

Thirty patients with sepsis who were admitted to the intensive care unit of the General Hospital of Ningxia Medical University, China, between 2019 and 2021 with indications for nutritional support, were randomly assigned to one of three different modalities of nutritional support. For 5 d, nutritional support was administered to each patient using one of the following modalities: TEN group, total parenteral nutrition (TPN group), and supplemental parenteral nutrition (SPN group). Blood and stool specimens were collected before and after nutritional support was administered, and changes in gut microbiota, SCFAs, and immune and nutritional indicators were detected and compared among the three groups.

Research results

Patients were assessed before and after the administration of nutritional support. The following differences were observed in the three groups after nutritional support: (1) Differences in the gut bacteria (Enterococcus increased in the TEN group, Campylobacter decreased in the TPN group, and Dialister decreased in the SPN group); (2) different trends in SCFA concentrations (increase in SCFAs in the TEN group except for caproic acid, improvement of acetic and propionic acid only in the TPN group, and decreasing trend in SCFA production in the SPN group); and (3) significant improvement in the nutritional and immunological indicators in the TEN and SPN groups, with improvement in immunoglobulin G levels only in the TPN group. Furthermore, and significant correlations were found

between the gut bacteria, SCFAs, and nutritional and immunological indicators.

Research conclusions

Our results indicate that TEN is the optimal modality for early nutritional support in patients with sepsis from the perspective of intestinal microecology.

Research perspectives

Future research should focus on how different nutritional support modalities affect the structural and metabolic changes in gut microbiota composition and the underlying mechanisms. Our research group will explore these questions in a large scale clinical trial with a longer intervention time.

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FOOTNOTES

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SYSTEMATIC REVIEWS

Occam's razor or Hickam's dictum-COVID-19 is not a textbook aetiology of acute pancreatitis: A modified Naranjo Score appraisal

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Abstract

BACKGROUND

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe disease. During the coronavirus disease 2019 (COVID-19) pandemic, numerous reports of AP have been published, with most authors concluding a causal relationship between COVID-19 and AP. Retrospective case reports or small case series are unable to accurately determine the cause-effect relationship between COVID-19 and AP.

AIM

To establish whether COVID-19 is a cause of AP using the modified Naranjo scoring system.

METHODS

A systematic review was conducted on PubMed, World of Science and Embase for articles reporting COVID-19 and AP from inception to August 2021. Exclusion criteria were cases of AP which were not reported to be due to COVID-19 infection, age < 18 years old, review articles and retrospective cohort studies. The original 10-item Naranjo scoring system (total score 13) was devised to approximate the likelihood of a clinical presentation to be secondary to an adverse drug reaction. We modified the original scoring system into a 8-item modified Naranjo scoring system (total score 9) to determine the cause-effect relationship between COVID-19 and AP. A cumulative score was decided for each case presented in the included articles. Interpretation of the modified Naranjo scoring system is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable cause.

RESULTS

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67 articles were included in the final analysis, with 76 patients which had AP reported to be due to COVID-19. The mean age was 47.8 (range 18-94) years. Majority of patients (73.3%) had ≤ 7 d between onset of COVID-19 infection and diagnosis of AP. There were only 45 (59.2%) patients who had adequate investigations to rule out common aetiologies (gallstones, choledocholithiasis, alcohol, hypertriglyceridemia, hypercalcemia and trauma) of AP. Immunoglobulin G4 testing was conducted in 9 (13.5%) patients to rule out autoimmune AP. Only 5 (6.6%) patients underwent endoscopic ultrasound and/or magnetic resonance cholangiopancreatogram to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum. None of the patients had other recently diagnosed viral infections apart from COVID-19 infection, or underwent genetic testing to rule out hereditary AP. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP.

CONCLUSION

Current evidence is weak to establish a strong link between COVID-19 and AP. Investigations should be performed to rule out other causes of AP before establishing COVID-19 as an aetiology.

Key Words: COVID-19; Infections; Pancreatic diseases; Pancreatitis; Post-acute COVID-19 syndrome

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Core Tip: Numerous reports of acute pancreatitis (AP) have been published during the coronavirus disease 2019 (COVID-19) pandemic, citing COVID-19 as an aetiology of AP. However, COVID-19 has not been well-established to be a cause of AP. A total of 76 patients were included in our systematic review and were assessed using the modified Naranjo score; there were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP. The link between COVID-19 and AP is weak based on current literature; COVID-19 should still remain a diagnosis of exclusion for AP until further evidence.

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INTRODUCTION

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe, with an incidence of 50-80 per 100000 population[1]. Gallstone disorders and alcohol abuse remain the two commonest global causes of AP[2]. In rare circumstances, AP may be triggered by viral or parasitic infections[3,4]. Recently, there have been reports of AP caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of the time of this article, the coronavirus disease 2019 (COVID-19) pandemic is responsible for over 642 million infections and 6.6 million deaths worldwide[5]. While COVID-19 is primarily a respiratory disease, patients with COVID-19 infection may experience extra-pulmonary symptoms[6]. There has been an increase in reports of autoimmune and inflammatory conditions attributed to SARS-CoV-2[7], one example of which is AP. While there have been sporadic case reports and attempts at literature reviews on the potential cases of COVID-19-induced AP, the significant increase in cases reported raises concerns regarding COVID-19 as a definitive causal etiology for AP rather than an epiphenomenon[8].

The distinction between "association" *vs* "causation" can only be derived by prospective longitudinal studies involving a large population or by rigorous statistical analysis of large datasets and registries. It remains unproven if published reports of AP (effect) are beyond doubt due to COVID-19 infection (causal etiology). This is similar to determining whether symptoms experienced following ingestion of a new medication may be coined as an "adverse drug reaction" and attributed to that drug. Naranjo *et al* [9] designed a questionnaire to determine the likelihood of an adverse drug reaction (effect) due to a drug (causal etiology) rather than associated confounding factors[9]. Recently, we have reported the utility of modified Naranjo score to diagnose AP in patients prescribed with sulphasalazine[10]. This is a novel study which aims to systematically review case series and/or reports on COVID-19-induced AP

and assess them using the modified Naranjo score to determine whether there is a cause-effect relationship.

MATERIALS AND METHODS

Study selection and search strategy

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines[11]. A literature search was performed on PubMed, World of Science and Embase for articles on COVID-19 and AP from inception till August 8, 2021. The following search terms were used: ((“COVID-19” OR “Coronavirus 19” OR “SARS-CoV-2”) AND (“Pancreatitis” OR “Acute Pancreatitis” OR “Acute Edematous Pancreatitis” OR “Pancreatic Inflammation”)); MeSH terms were used where available. Inclusion criteria were case series and/or case reports on AP, where the aetiology was thought to be COVID-19 infection. Exclusion criteria were (1) reports on AP but aetiology was not attributed to COVID-19 infection; (2) cases with patients < 18 years old; and (3) based on article type (non-English language, review articles and retrospective cohort and/or case-control studies). After removing of duplicates, three authors (Tang ZJT, Chua BQY, and Lim PK) independently screened the articles for potential inclusion by title and abstract. Subsequently, the full texts of screened studies were obtained and reviewed for eligibility. As some case reports may not have abstracts published, full texts were reviewed if abstracts were not available. Conflicts were resolved by appeal to the senior author.

The modified Naranjo score

The original Naranjo score was described by Naranjo *et al*[9] in 1981 as a means of approximating the likelihood of attributing a patient’s presentation to a medication and labelling it as an adverse drug reaction[9]. It consists of ten closed-ended questions with the options for “Yes”, “No,” and “Do not know”, each with varying points allocated from “- 1” to “+ 2”. The total score is 13 and may be interpreted as such: ≤ 0: Doubtful, 1-4: Possible; 5-8: Probably and ≥ 9: Definite. Existing studies have modified the Naranjo score to determine the cause-effect relationship in various pathologies[10]. In this review, we propose a modification to the Naranjo score to determine the likelihood of COVID-19-induced AP (Table 1). This is a 8-item scoring system with a total of 9 points; a cumulative score was calculated for each case, and a probability classification was assigned based on their score: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable.

Our modified version of the Naranjo score retained some of the questions used in the original study: Whether reports suggest a causal relationship, if the resolution of COVID-19 led to the resolution of AP, and ruling out alternative causes of AP. Common causes of AP were defined as gallstones, choledocholithiasis, alcohol, hypertriglyceridaemia, hypercalcaemia or trauma. Investigations for these included history and physical examination (for alcohol and trauma), biochemical investigations (for hypertriglyceridaemia and hypercalcemia), imaging studies (ultrasonography and/or computed tomography of the abdomen and pelvis) (for gallstones or choledocholiathisis) and endoscopic retrograde cholangiopancreatogram for choledocholithiasis in the presence of abnormal liver function test and/or biliary dilation. The original Naranjo score included the need for drug challenge test or placebo, but this was not appropriate for this study, as a “drug challenge” would imply re-introduction of COVID-19 infection and identifying if the patient had recurrence of AP. Instead, whether the resolution of infection led to the resolution of acute pancreatitis was included as a criterion.

We additionally included questions on whether there is a cause-effect temporal relationship and a more in-depth approach in ruling out the other aetiologies of AP. This included ruling out autoimmune pancreatitis with testing for serum Immunoglobulin G4 (IgG4)[12], or whether an endoscopic ultrasound (EUS) or magnetic resonance cholangiopancreatography (MRCP) was performed to rule out occult microlithiasis, pancreatic malignancy or pancreatic divisum[13]. While genetic testing to rule out hereditary pancreatitis is a consideration[14], this was omitted in our modified Naranjo score as none of the included articles performed genetic tests for confirming/excluding hereditary pancreatitis. Additionally, there are no strict recommendations on the exact indications for genetic counselling and/or testing in AP, limiting its utility for inclusion in our proposed scoring system[15].

Data extraction

Three authors (Tang ZJT, Chua BQY, and Lim PK) performed all data extraction independently using the systematic review management tool Covidence (https://www.covidence.org). The following data were extracted: Year of study, age, and gender of the patient, and features relevant to the modified Naranjo score, as shown in Table 1.

RESULTS

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67

Table 1 Modified Naranjo Score used to grade the included studies with respective points allocated to each criterion

Criteria	Yes	No	Unsure
Are there published reports of the COVID-19 causing acute pancreatitis?	+ 1	0	0
Was there short latency (≤ 7 d) between the onset of infection and the diagnosis of acute pancreatitis?	+ 2	- 1	0
Was there a temporal relationship (≤ 1 mo) between onset of infection and onset of acute pancreatitis symptoms?	+ 1	- 1	0
Did the acute pancreatitis resolve following resolution of the infection?	+ 1	- 1	0
Were all commonly known causes of acute pancreatitis ruled out? (*e.g.*, gallstones/choledocholithiasis, alcohol, hypertriglyceridaemia, hypercalcaemia, ERCP, trauma)	+ 1	- 1	0
Was a serum IgG4 level checked? (To rule out autoimmune pancreatitis)	+ 1	0	0
Does the patient have or was the patient recently diagnosed with an infection (other than COVID-19) which could cause pancreatitis?	- 1	+ 1	0
Was an EUS and/or MRCP performed? (*e.g.*, to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum)	+ 1	- 1	0

COVID-19: Coronavirus disease 2019; IgG4: Immunoglobulin G4; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; MRCP: Magnetic resonance cholangiopancreatography.

articles were included in the final analysis (Figure 1). There was a total of 76 patients which had AP reported to be due to COVID-19. Data extracted from all cases were reported in Table 2[16-82]. Seventy-six patients with a mean age of 47.8 years (range 18-94) were reported. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP (Figure 2A). Majority of patients (73.7%, n = 56/76) had a short duration of latency (≤ 7 d) between the onset of infection and AP. Most reports did not explore the temporal relation between COVID-19 and AP; only 20 (26.3%) patients were described to have a temporal relationship between COVID-19 and AP. Common aetiologies of AP were ruled out in 45 (59.2%) patients. Serum IgG4 Levels were tested to rule out autoimmune pancreatitis in 9 (13.6%) patients. None of the patients were recently diagnosed with an infection known to cause AP, *e.g.*, Coxsackie virus, cytomegalovirus, or herpes simplex virus. Only a minority (6.6%, n = 5/76) underwent either EUS or ERCP. None of the patients underwent genetic testing, *e.g.*, *SPINK-1*, to rule out hereditary AP.

DISCUSSION

AP is a common cause of acute abdominal pain and hospital admissions and present as a disease spectrum[83]. In view of the COVID-19 pandemic, many retrospective case reports suggesting COVID-19 as a cause of AP have been published. It is an obligation to critically appraise these reports to define the strength of the association and evaluate if the co-occurrence is a mere association or actual causation. This novel study adopted the modified Naranjo scale and concluded the cause-effect relationship between COVID-19 and AP in most reports as doubtful (42.1%) or possible (51.3%).

While gallstones and alcohol consumption remain the most common aetiologies, in rare situations, viral infections have been reported to cause AP[3,4]. However, this should be a diagnosis of exclusion after ruling out of common aetiologies for AP. In our review, the reason why strong conclusions could not be drawn for most cases was the absence of serum IgG4 Levels (86.4%) to rule out autoimmune AP (score of + 1) and EUS or MRCP (93.4%) to rule out structural causes (*e.g.* occult microlithiasis or pancreatic divisum) (score of +1). Authors reporting further cases of AP where COVID-19 is suggested as an aetiology should perform these tests to rule out other aetiologies of AP prior to conclusion of COVID-19-induced AP. While the results do not confirm AP as a possible aetiology of COVID-19, it is still prudent to consider COVID-19 when patients present with AP, especially if there are concurrent clinical features of COVID-19 with temporal relation of symptoms and if other common causes are ruled out.

The current literature is torn on the idea that the SARS-CoV2 virus could have induced the recent cases of AP, where some propose a direct causal relationship and others claim the SARS-CoV2 virus as a bystander in the instances of idiopathic AP[84-86]. For the former, two possible mechanisms have been described: The direct effect of the SARS-CoV2 RNA on pancreatic tissue due to viral tropism and the indirect effect due to microthrombi formation. Regarding the direct mechanism, the expression of the angiotensin converting enzyme 2 (ACE2) protein on the SARS-CoV2 virus primes its entry into the pancreas. This expression of the ACE2 protein was similarly found in the islet and exocrine tissue microvasculature and in a subset of pancreatic ducts, as well as the TMPRSS2 proteins found in the ductal cells[84]; this suggests entry of SARS-CoV2 into pancreatic tissue resulting in AP. Regarding the

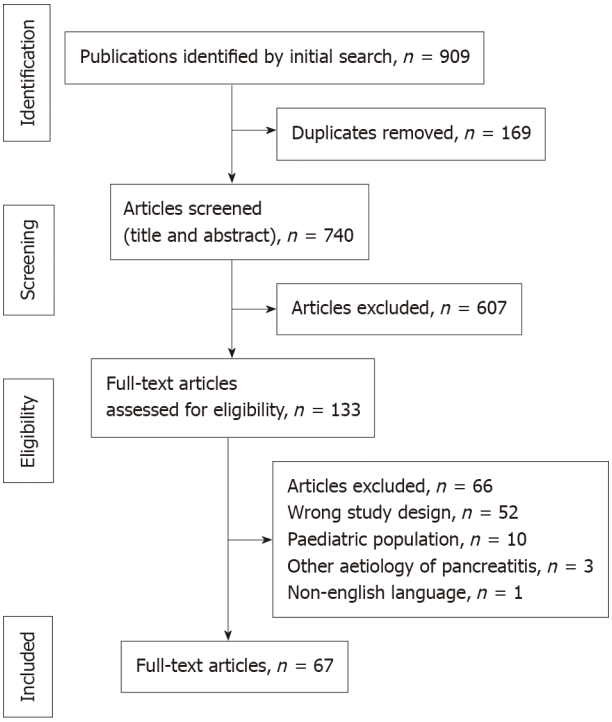
Table 2 Summary of all the included case reports (*n* = 76 patients) with respective patient demographics, modified Naranjo Score and interpretation

No	First author	Year	Patient age/Sex	Scoring								Summed score	Result[1]
1	Acherjya *et al*[16]	2020	57/F	1	2	1	1	1	0	1	-1	6	Possible
2	Al-Douri *et al*[17]	2020	45/F	1	2	1	0	1	0	1	-1	5	Possible
3	Al-Harmi *et al*[18]	2021	52/F	1	0	1	0	1	0	1	-1	3	Doubtful
4	Ali *et al*[19]	2021	53/M	1	0	1	0	-1	0	1	-1	1	Doubtful
5	Aloysius *et al*[20]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
6	Alves *et al*[21]	2020	56/F	1	2	1	1	1	0	1	1	8	Probable
7	Alwaeli *et al*[22]	2020	30/M	1	2	1	0	1	0	1	-1	5	Possible
8	Amé and Balderramo[23]	2022	42/F	1	2	1	0	1	0	1	-1	5	Possible
9			65/F	1	0	1	0	1	1	1	1	6	Possible
10	Anand *et al*[24]	2020	59/F	1	0	1	0	1	0	1	-1	3	Doubtful
11	Arjun *et al*[25]	2020	34/M	1	2	1	1	1	1	1	-1	7	Probable
12	Bains *et al*[26]	2020	68/M	1	2	1	0	-1	0	1	1	5	Possible
13	Bokhari and Mahmood[27]	2020	32/M	1	2	1	0	1	0	1	-1	5	Possible
14	Bouali *et al*[28]	2021	60/F	1	2	1	0	-1	0	1	-1	3	Doubtful
15	Brikman *et al*[29]	2020	61/M	1	0	1	0	1	0	1	-1	3	Doubtful
16	Canastar *et al*[30]	2020	64/M	1	2	1	0	-1	0	1	-1	3	Doubtful
17	Chandra *et al*[31]	2021	53/M	1	2	1	0	-1	0	1	-1	3	Doubtful
18	Cheung *et al*[32]	2020	38/M	1	2	1	0	1	1	1	1	8	Probable
19	Chivato *et al*[33]	2021	55/M	1	2	1	1	1	0	1	1	8	Probable
20	Dufani *et al*[34]	2020	27/F	1	2	1	0	1	0	1	-1	5	Possible
21	Elhence *et al*[35]	2020	31/F	1	0	1	0	-1	0	1	-1	1	Doubtful
22			40/M	1	0	1	0	-1	0	1	-1	1	Doubtful
23			42/M	1	0	1	0	-1	0	1	-1	1	Doubtful
24	Fernandes *et al*[36]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
25	Fiore *et al*[37]	2021	42/M	1	2	1	0	1	0	1	-1	5	Possible
26			70/M	1	0	1	0	1	0	1	-1	3	Doubtful
27	Gadiparthi *et al*[38]	2020	40/M	1	2	1	1	-1	0	1	-1	4	Possible
28	Gadiparthi *et al*[39]	2021	74/F	1	2	1	0	1	0	1	-1	5	Possible
29	Gonzalo-Voltas *et al*[40]	2020	76/F	1	2	1	1	1	0	1	-1	6	Possible
30	Gupta *et al*[41]	2021	25/F	1	2	1	1	1	0	1	-1	6	Possible
31	Hadi *et al*[42]	2020	47/F	1	2	1	0	-1	0	1	-1	3	Doubtful
32			68/F	1	2	1	0	-1	0	1	-1	3	Doubtful
33	Hanif *et al*[43]	2021	30/F	1	2	1	0	-1	0	1	-1	3	Doubtful
34	Hassani *et al*[44]	2020	78/F	1	2	1	0	1	0	1	-1	5	Possible
35	Ibrahim *et al*[45]	2020	33/M	1	2	1	0	-1	0	1	-1	3	Doubtful
36	Jespersen Nizamic *et al*[46]	2020	49/W	1	0	1	0	1	0	1	-1	3	Doubtful
37	Kandasamy[47]	2020	45/F	1	2	1	1	1	0	1	-1	6	Possible
38	Karimzadeh *et al*[48]	2020	65/F	1	2	1	0	-1	0	1	-1	3	Doubtful
39	Kataria and Sharif[49]	2020	49/F	1	2	1	1	1	0	1	-1	6	Possible
40	Kolhe *et al*[50]	2020	19/F	1	2	1	1	1	0	1	-1	6	Possible

41	Kumaran *et al*[51]	2020	67/F	1	2	1	1	1	1	1	-1	7	Probable
42	Kurihara *et al*[52]	2020	55/M	1	0	1	0	1	0	1	-1	3	Doubtful
43	Lakshmanan and Malik[53]	2020	68/M	1	2	1	0	1	0	1	-1	5	Possible
44	Maalouf *et al*[54]	2021	62/M	1	2	1	0	1	0	1	-1	6	Possible
45	Mazrouei *et al*[55]	2020	24/M	1	2	1	0	-1	0	1	-1	3	Doubtful
46	Meireles *et al*[56]	2020	36/F	1	0	1	1	1	1	1	-1	5	Possible
47	Merza *et al*[57]	2020	57/M	1	2	1	0	-1	0	1	-1	3	Doubtful
48			70/M	1	0	1	0	-1	0	1	-1	1	Doubtful
49	Meyers *et al*[58]	2020	67/M	1	2	1	0	-1	1	1	-1	4	Possible
50	Miao *et al*[59]	2020	26/F	1	2	1	0	-1	0	1	-1	3	Doubtful
51	Mobin *et al*[60]	2020	18/M	1	2	1	0	1	0	1	-1	5	Possible
52			66/M	1	2	1	1	-1	0	1	-1	4	Possible
53	Mohammadi Arbati and Molseghi [61]	2021	28/M	1	2	1	0	-1	0	1	-1	3	Doubtful
54	Muhammad Abrar Jeelani *et al*[62]	2021	24/M	1	0	1	1	1	0	1	-1	4	Possible
55	Naqvi *et al*[63]	2020	69/F	1	2	1	0	1	0	1	-1	5	Possible
56	Narang *et al*[64]	2021	20/F	1	2	1	0	1	1	1	-1	6	Possible
57	Patnaik *et al*[65]	2020	29/M	1	2	1	0	1	0	1	-1	5	Possible
58	Pinte and Baicus[66]	2020	47/M	1	0	1	0	1	0	1	-1	4	Possible
59	Purayil *et al*[67]	2020	58/M	1	2	1	0	-1	0	1	-1	3	Doubtful
60	Rabice *et al*[68]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
61	Rotar *et al*[69]	2020	39/M	1	2	1	0	-1	0	1	-1	3	Doubtful
62	Sandhu *et al*[70]	2021	25/F	1	2	1	0	1	0	1	-1	5	Possible
63	Shinohara *et al*[71]	2020	58/M	1	0	1	1	-1	0	1	-1	2	Doubtful
64	Simou *et al*[72]	2020	67/NM	1	0	1	0	-1	0	1	-1	1	Doubtful
65	Singh and Kharoud[73]	2020	94/F	1	2	1	0	1	1	1	-1	6	Possible
66			58/F	1	0	1	0	1	1	1	-1	4	Possible
67	Srinivasan *et al*[74]	2021	52/F	1	2	1	0	-1	0	1	-1	3	Doubtful
68	Tollard *et al*[75]	2021	32/F	1	2	1	0	1	0	1	-1	5	Possible
69	Tomasi *et al*[76]	2021	31/M	1	2	1	0	1	0	1	-1	5	Possible
70	Truscello *et al*[77]	2020	71/M	1	2	1	0	-1	0	1	-1	3	Doubtful
71	Wang *et al*[78]	2020	42/M	1	2	1	0	1	0	1	-1	5	Possible
72			35/M	1	2	1	1	1	0	1	-1	6	Possible
73	Wifi *et al*[79]	2021	72/F	1	2	1	0	-1	0	1	-1	3	Doubtful
74	Yamamoto *et al*[80]	2021	70/F	1	0	1	0	-1	0	1	-1	1	Doubtful
75	Zeng *et al*[81]	2020	36/M	1	2	1	0	0	0	1	-1	4	Possible
76	Zielecki *et al*[82]	2020	38/M	1	0	1	1	-1	0	1	0	3	Doubtful

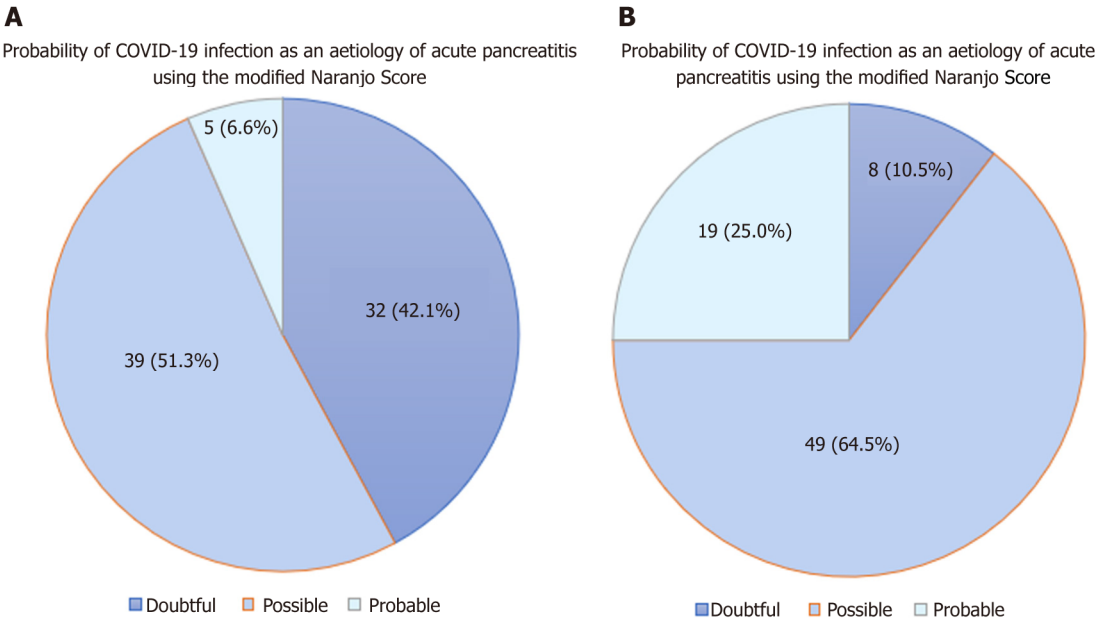
[1]Interpretation of the modified Naranjo score is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable.
F: Female; M: Male; NM: Not mentioned.

indirect mechanism, SARS-CoV2 causes microthrombi formation due to its ability to invade endothelial cells *via* the ACE2 protein. This phenomenon is also observed in other virus infections, such as viral hepatitis[85]. Microthrombus in vasculature triggers hypoperfusion of the pancreas, resulting in ischemic pancreatitis. However, the above theories are merely conjectures made from parallel existing conditions superimposed on an under-researched virus. The possibility of COVID-19 being a pure bystander in the formation of AP also exists. This may be a situation of Occam's razor– where we look



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Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses figure showing the study selection process.



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Figure 2 Graphical representation of the probability of coronavirus disease 2019 as an aetiology of acute pancreatitis. A: Using the modified Naranjo score proposed; B: Using the modified Naranjo score, if a score of 1 was subtracted from the minimum cut-off for each category, where ≤ 2 is for doubtful, 3-5 is for possible and ≥ 6 is for probable significance. COVID-19: Coronavirus disease 2019.

to connect the dots between a relatively new disease and the patients' manifestations– *vs* a pure case of Hickam's dictum– where a patient happens to present with two mutually exclusive conditions. After all, it must be noted that idiopathic AP is a function of diagnostic workup efforts[86]. The lack of serological tests for IgG4, MRCP scan, and EUS imaging could inflate the association between COVID-19 and AP. As randomized studies cannot be conducted to establish COVID-19 as an aetiology for AP, critical appraisal of retrospective data is essential to discern association from causation.

The temporal relation of events is one of the important determinants of distinguishing causation from the association. Many authors did not explicitly mention a temporal relation of onset or resolution of COVID-19 and AP. While COVID-19 infections primarily involve the respiratory system, multi-systemic involvement have been reported. Since COVID-19 is a prerequisite for COVID-19 associated AP, we shall discuss the respiratory and imaging features first. COVID-19-induced AP may manifest in the absence of any respiratory symptoms or radiological evidence of lung involvement. Purayil *et al*[67] reported a 58-year-old male presenting with abdominal pain; a polymerase chain reaction (PCR) for COVID-19 was only done in response to his chest x-ray, which revealed bilateral infiltrates in the absence of respiratory symptoms[67]. A chest x-ray is routinely performed in patients with acute abdomen to rule out free air under the diaphragm secondary to hollow viscus perforation, which also can cause hyperamylasaemia and remain an important differential diagnosis of AP[87]. Additionally, chest x-ray findings form a part of scoring systems to predict severity and clinical outcomes in AP patients[88,89]. In the patient reported by Purayil *et al*[67], the chest x-ray was sensitive to detect COVID-19 changes and complemented the diagnostic work-up for epigastric pain. As most patients with AP will have a chest x-ray performed and due to the widespread prevalence of COVID-19 in the community, it is not possible to ascertain if COVID-19 resulted in AP or if the two diseases merely occurred simultaneously yet independently of each other.

The prevalence of gastrointestinal symptoms in COVID-19 infected patients ranges from 3.0%-79%, of which only 2.2%-6.0% of patients present with abdominal pain[90-92]. Abdominal pain is the most common symptom of AP and acute epigastric pain is one of the key diagnostic criteria of AP. Thus it remains unclear if gastrointestinal manifestations of COVID-19 are secondary to AP. Though resuscitation and early management of AP patients are not determined by aetiology, definite management for prevention of future occurences is determined by aetiology. Thus, knowledge of COVID-19 as an aetiology of AP is important as it stops the pursuit of aetiology identification, guides physician on the counselling of their patients, and impacts management decision for cholecystectomy. In patients with mild biliary pancreatitis, index admission laparoscopic cholecystectomy is considered good clinical practice while surgery should be delayed in patients with COVID-19 infection to minimize risk to healthcare workers and reduce patient morbidity[93,94]. Shao *et al*[95] analysed 589 patients with COVID-19 infection prior to surgery and concluded that postoperative mortality was nearly 6 times higher for patients infected with COVID-19 within 2 wk before surgery when adjusting for patient and procedure level factors[95]. Furthermore, delaying cholecystectomy in patients with biliary pancreatitis could increase the risk of future biliary events[88]. Thus, determining the aetiology of AP is essential as it impacts clinical decisions.

In addition to abdominal pain and imaging features, it is important to discuss the role of serum enzymes. While both amylase and lipase are usually measured in patients presenting with acute abdomen suggesting AP, the timing at which they rise can be cross-referenced to the time at which COVID-19 infection was diagnosed, which may shed some light on whether COVID-19 is a cause of AP, or merely coincidental. Amylase tends to rise within 3 to 6 h of AP and persists for up to 5 d. However, serum amylase has a relatively short half-life of 12 h and may return to normal limits within a day. Serum lipase rises similarly within 3 to 6 h, peaks at 24 h, and persists for 8 to 14 d. Hence, a patient with raised amylase or lipase alongside a concomitant COVID-19 infection before this time span of 12-24 h may still have AP caused by COVID-19. This clarity blurs if we consider the virus's incubation period and heterogeneity in the clinical presentation of both COVID-19 and AP. Stephens *et al*[96] reported 234 COVID-19 positive patients in the critical care unit, of which 52 (22.2%) patients had peak amylase three times the upper limit of normal, of which only 4 (1.7%) met the revised Atlanta criteria for diagnosis of AP[96]. Furthermore, some authors report COVID-19 associated hyperamylasemia secondary to pancreatic injury which does not amount to clinical AP[97,98]. This could be attributed to amylase release from other viscera like the gastrointestinal tract or elevated serum levels due to reduced renal excretion as a result of critical illness-related kidney injury[96]. A contrast-enhanced imaging remains an essential tool to diagnose AP, but it would not establish COVID-19 as a causative aetiology.

As the temporal occurrence of events (*e.g.* onset of abdominal pain, chest x-ray imaging, serum enzymes, and abdominal imaging) do not aid the distinction of causation or association, a different approach is essential. This situation is similar to the dilemma of attributing a clinical presentation towards an adverse drug reaction. The Naranjo score is a useful tool that aids clinical judgment in differentiating the cause-effect relationship between a drug and its possible adverse drug reaction[9]. While useful in determining the causality, it should be noted that the scale does not offer prognostic information. In addition, the Naranjo score includes isolating toxic concentrations of drugs in body fluids, clinical response to placebo administration, and a drug rechallenge to assess symptoms occurrence. While most authors report positive COVID-19 rapid antigen test and PCR, the response to placebo and re-infection are not reported. It is not possible to assess if the patients were re-infected with COVID-19 (after reporting of the case) with repeat AP and unethical to do this experimentally. Thus, no comment can be made on whether a 're-challenge' would bring about similar symptoms. If these parameters are scored negative, many cases would be disadvantaged from the strength of causation, and thus, a modification of the Naranjo score that excludes placebo administration and re-infection was considered essential to determine COVID-19 as a cause of AP fairly. This approach has been reported in the past for sulphasalazine-induced AP and prednisolone induced pneumatosis coli[10,99]. Using this

strategy, we determined the strength of association and possible causation as doubtful, possible or probable. Other established methods of determining a causal relationship between various aetiological agents and AP include the Badalov categorisation[100]. The Badalov categorisation was similarly designed to investigate association between drugs and adverse drug reactions; this involves assigning drugs into 5 categories (Class Ia, Ib, II, III and IV) based on the number of case reports published, drug rechallenge, latency and whether alternative causes were excluded. However, we have chosen to adopt the Naranjo score as it allows for a case-by-case evaluation of each report as opposed to a blanket categorization of COVID-19 as a possible aetiological agent of AP. Secondly, the Naranjo score also provides a better idea on the degree of association by generating data on a numerical scale which allows for in-depth analysis. Centers should continue reporting such occurrences of COVID-19-induced pancreatitis and consider incorporating our modified Naranjo score; artificial intelligence methods may subsequently be used to diagnose COVID-19-induced pancreatitis[101,102].

However, our study has its limitations. Firstly, given the nature of our study, prospective studies and systematic reviews were not analysed as they lack individual patient data. Additionally, interpretation of this study is limited by the small sample size of 76 patients. Secondly, a majority of included case reports date after the introduction of the COVID-19 vaccine. Many reports did not mention the vaccination status of the patient; COVID-19 vaccination has also been reported as a potential cause of AP which adds on to the dilemma[24,103]. Thirdly, with the advent of novel drugs used in COVID-19 treatment, the possibility of drug-induced AP needs to be considered as a differential diagnosis. Remdesivir is an antiviral drug widely used in the management of COVID-19 and it is increasingly reported to cause pancreatic injury with associated hyperamylasaemia as well as AP[104,105]. Fourthly, authors may not have reported cases where patients were reinfected by COVID-19. This is a potential limitation in the calculation of the modified Naranjo score. Fifthly, the determination of the criteria for "doubtful", "possible" and "probable" is arbitrary. If we subtract one point from the minimum of each category (*i.e.* ≤ 2 for doubtful, 3-5 for possible and ≥ 6 for probable) to increase the causation strength, this would result in 8 (compared to 32) patients, 49 patients (compared to 39), and 19 patients (compared to 5) respectively (Figure 2A and B). As the median score was 4, if we use it as the cut-off, 32 patients (score < 4) will be categorized as "less likely" and 44 patients (score ≥ 4) as "more likely" to have COVID-19-induced AP. Thus, the assignment of scores, though partly arbitrary, the fact still prevails that existing reports have doubt about COVID-19 infection causing AP. Lastly, while some of the screened cases reported fatalities[13], our study did not assess the severity and mortality of COVID-19 induced AP.

CONCLUSION

The use of our proposed modified Naranjo score may help to determine whether COVID-19 is a likely aetiology of AP and may assist clinicians in making useful clinical decisions. The current evidence is weak to establish a strong causal link between COVID-19 and AP, and more evidence is necessary before COVID-19 should be incorporated as a "textbook aetiology" of AP.

ARTICLE HIGHLIGHTS

Research background

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe disease. During the coronavirus disease 2019 (COVID-19) pandemic, numerous reports of AP have been published, with most authors concluding a causal relationship between COVID-19 and AP.

Research motivation

Published reports or case series of COVID-19-induced AP are retrospective in nature and are unable to accurately determine the cause-effect relationship between COVID-19 and AP.

Research objectives

This study aims to establish whether COVID-19 is a cause of AP by proposing a scoring system *i.e.* the modified Naranjo scoring system.

Research methods

A systematic review was conducted on PubMed, World of Science and Embase for articles reporting COVID-19 and AP from inception to August 2021. Exclusion criteria were cases of AP which were not reported to be due to COVID-19 infection, age < 18 years old, review articles and retrospective cohort studies. The original 10-item Naranjo scoring system (total score 13) was devised to approximate the likelihood of a clinical presentation to be secondary to an adverse drug reaction. We modified the

original scoring system into a 8-item modified Naranjo scoring system (total score 9) to determine the cause-effect relationship between COVID-19 and AP. A cumulative score was decided for each case presented in the included articles. Interpretation of the modified Naranjo scoring system is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable cause.

Research results

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67 articles were included in the final analysis, with 76 patients which had AP reported to be due to COVID-19. The mean age was 47.8 (range 18-94) years. Majority of patients (73.3%) had ≤ 7 d between onset of COVID-19 infection and diagnosis of AP. There were only 45 (59.2%) patients who had adequate investigations to rule out common aetiologies (gallstones, choledocholithiasis, alcohol, hypertriglyceridemia, hypercalcemia and trauma) of AP. Immunoglobulin G4 testing was conducted in 9 (13.5%) patients to rule out autoimmune AP. Only 5 (6.6%) patients underwent endoscopic ultrasound and/or magnetic resonance cholangiopancreatogram to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum. None of the patients had other recently diagnosed viral infections apart from COVID-19 infection, or underwent genetic testing to rule out hereditary AP. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP.

Research conclusions

Current evidence is weak to establish a strong link between COVID-19 and AP. Investigations should be performed to rule out other causes of AP before establishing COVID-19 as an aetiology.

Research perspectives

The use of our proposed modified Naranjo score may help to determine whether COVID-19 is a likely etiology of AP and may assist clinicians in making useful clinical decisions.

FOOTNOTES

Author contributions: Teng TZJ, Chua BQY and Lim PK contributed equally to this work; Teng TZJ, Chua BQY and Lim PK performed the literature search, data collection and wrote the manuscript; Chan KS wrote the manuscript and revised the manuscript; Shelat VG conceptualised the study design and idea, and revised the manuscript; All authors have read and approve the final manuscript.

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REVIEW

Insight into the liver dysfunction in COVID-19 patients: Molecular mechanisms and possible therapeutic strategies

Naina Khullar, Jasvinder Singh Bhatti, Satwinder Singh, Bhawana Thukral, P Hemachandra Reddy, Gurjit Kaur Bhatti

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Abstract

As of June 2022, more than 530 million people worldwide have become ill with coronavirus disease 2019 (COVID-19). Although COVID-19 is most commonly associated with respiratory distress (severe acute respiratory syndrome), meta-analysis have indicated that liver dysfunction also occurs in patients with severe symptoms. Current studies revealed distinctive patterning in the receptors on the hepatic cells that helps in viral invasion through the expression of angiotensin-converting enzyme receptors. It has also been reported that in some patients with COVID-19, therapeutic strategies, including repurposed drugs (mitifovir, lopinavir/ritonavir, tocilizumab, *etc.*) triggered liver injury and cholestatic toxicity. Several proven indicators support cytokine storm-induced hepatic damage. Because there are 1.5 billion patients with chronic liver disease world-wide, it becomes imperative to critically evaluate the molecular mechanisms concerning hepatotropism of COVID-19 and identify new potential therapeutics. This review also designated a comprehensive outlook of comorbidities and the

impact of lifestyle and genetics in managing patients with COVID-19.

Key Words: COVID-19; Liver damage; Pharmacotherapy; Cytokine storm; Molecular mechanisms

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Core Tip: Several review articles have contributed to the pathophysiology, therapeutic strategies, vaccine development, and clinical trials of coronavirus disease 2019 (COVID-19). Since the liver is the primary site of immune protein synthesis, any liver defect may compromise the immune system. Patients with chronic liver disease are at a higher risk of severe COVID-19. This review article demonstrated the pathophysiology and molecular mechanisms responsible for more severe outcomes in patients with hepatic defects. Further, we critically evaluated the molecular mechanisms concerning hepatotropism in patients with COVID-19, which could lead to the development of new therapeutics.

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INTRODUCTION

On December 31, 2019, reports surfaced of patients with an unusual severe acute respiratory syndrome (pneumonia) in Wuhan, China. On January 7, 2020, the causal agent responsible for the mysterious deaths was branded as coronavirus disease 2019 (COVID-19) by the International Classification Committee of Viruses[1]. A severe global pandemic was declared on March 11, 2020. Since then, there have been 623893894 confirmed cases of COVID-19, including 6553936 deaths as of October 21, 2022[2].

The clinical manifestation of COVID-19 is usually interpreted as severe lung infection (acute respiratory distress syndrome), causing turmoil in the patient's respiratory system and even death[3]. Many individuals admitted to intensive care units were known to have hepatic and heart-related complications[4]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in patients with chronic liver disease leads to an elevated and dysregulated immune response[5]. Even healthy individuals infected with COVID-19 displayed liver dysfunction, increasing morbidity and mortality of such patients[6].

Hepatologists have been working to deduce the relationship between COVID-19 and the liver. About 300 million patients with chronic liver diseases in China alone had severe COVID-19 symptoms[7]. It was advocated that SARS-CoV-2 invasion might trigger reactivation of existing liver disorders in the patient, causing hepatotoxicity[8]. In addition, hepatic patients are more prone to COVID-19 infection [9]. Some COVID-19 cases have reported multiorgan failure significantly increasing cytokine levels, including vascular endothelial growth factor, interleukin (IL)-6, macrophage inflammatory protein 1α, and macrophage inflammatory protein 1β[9-11].

Since the liver is a primary site of the synthesis of proteins associated with immunity, it inhibits infectious microbes from entering the bloodstream from the gut. Any liver defect would thus cause a compromised immune system[12]. Increased levels of hepatic biomarkers including alanine transaminase and aspartate transaminase indicate a close pathophysiological association between the liver and COVID-19. Obesity predisposes individuals to metabolic disorders, diabetes, and insulin resistance, which can lead to chronic liver disease and may culminate into cirrhosis, fibrosis, and even hepatocellular carcinoma[13]. This indicates a dire need to investigate the pathophysiology and molecular mechanisms responsible for hindering the immune system, which leads to more severe outcomes in patients with cardiovascular and hepatic defects[14]. In this article, we comprehensively evaluated the invasion and spread of SARS-CoV-2 as a tool to improve therapeutic strategies against liver damage in patients with COVID-19.

ORIGIN OF SARS-COV-2: A BOTTOM-FEEDER OPPORTUNIST

The beta coronavirus isolated from COVID-19 patients was identified using whole genome sequencing and matrix representation with parsimony phylogenetic analysis[15,16]. This virus is the closest relative of Rhinolophus affinis virus (bat CoV RaTG13) with more than 96% similarity[17,18]. Thus, the bat is

Table 1 Summary of severe acute respiratory syndrome coronavirus 2 strains and mutations that enhance viral entry into the host cell

Sr. No.	Strain	Mutation	Host entry	Location	Time of first report	Ref.
1	Alpha (B.1.1.7)	N501Y mutation on the RBD	The affinity between RBD and ACE2 is significantly increased	United Kingdom	December 2020	[40-42]
2	Beta (B.1.351)	N501Y mutation on the RBD N417/K848/Y501	The affinity between RBD and ACE2 is significantly increased	South Africa	December 2020	[41-43]
3	Gamma (P.1)	N501Y mutation on the RBD N417/K848/Y501	The affinity between RBD and ACE2 is significantly increased	Brazil	January 2021	[41,42, 44]
4	Delta (B.1.617.2)	Absence of N501Y mutation	No effect	India	December 2020	[41,42]
5	Omicron (B.1.1.529)	N501Y mutation on the RBD	The affinity between RBD and ACE2 is significantly increased	South Africa	November 2021	[41,42]

RBD: Receptor binding domain; ACE2: Angiotensin-converting enzyme 2.

hypothesized to be its most suitable natural host. The phylogenetic interrelationship is depicted in Figure 1. Whole genome studies revealed the presence of three SARS-CoV-2 strains (A, B, and C) among various human populations[19]. Originally strain A was found in the European population and was regarded as the ancestral strain closest to the bat coronavirus[20,21]. Strain B was primarily found in East Asian populations[22]. Strain C, however, was more common among Americans, thus defining and tracking their outbreak areas[23]. Although the phylogenetic relationship of SARS-CoV-2 is still under investigation, it is well established that the preferred reservoir host of this virus is the bat and was transferred to humans from consumption of wild bats[18,24,25]. These are undoubtedly among the most prevalent RNA viruses (positive sense) that can infect a wide range of hosts[6,26]. Whole genome sequencing demonstrated that SARS-CoV-2 is most closely related to the CoV RaTG13 virus[27,28].

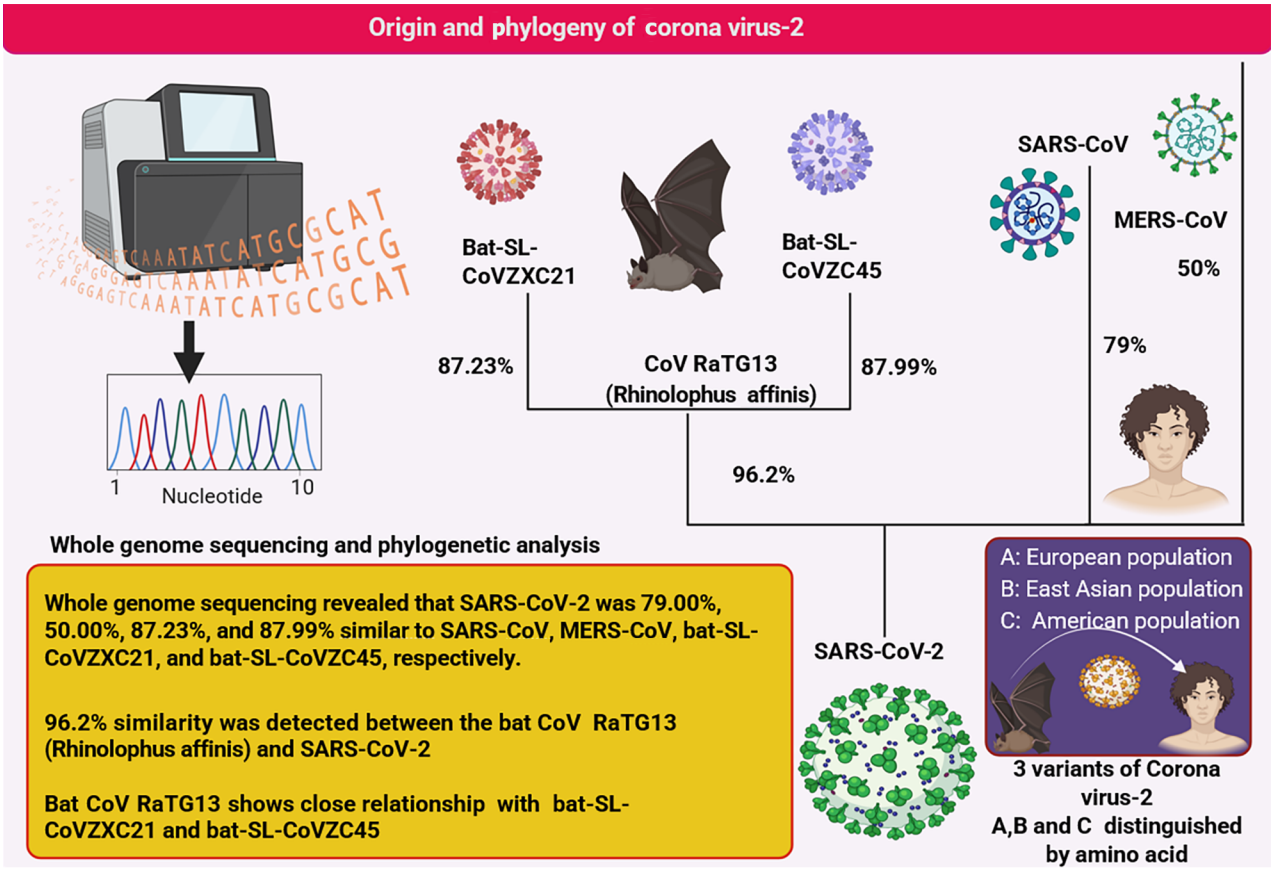
After almost three years since the discovery of SARS-CoV-2, the mechanism that allows SARS-CoV-2 to jump to a human host and invade hepatocytes remains unclear. Upregulated transaminases in patients with COVID-19 indicate a strong link between SARS-CoV-2 and liver injury[29,30]. The genetic algorithm for detecting recombination demonstrated that SARS-CoV-2 possesses a mosaic genome comparable to five coronaviruses (probable donor strains), namely Rhinolophus affinis RaTG13 coronavirus, Rhinolophus pusillus RpYN06, Rhinolophus pusillus BANAL-103, Rhinolophus malayanus RmYN02, and Rhinolophus malayanus BANAL-52[31-33]. The spike protein of SARS-CoV-2 has more similarity with Rhinolophus affinis RaTG13, and human angiotensin-converting enzyme 2 (ACE2) interaction is closely related to Rhinolophus malayanus BANAL-52. Another noteworthy feature is the absence of furin cleavage sites in all of these viruses[34].

SARS-CoV-2 has a deadly combination of disease severity and transmissibility[35,36]. Coronaviruses are remarkable entities (125 nm in size) with one of the heftiest viral RNA genomes, accounting for 30000 nucleotides and equipped with an extraordinary ability to correctly repair drug-induced mutations[37]. The chances of zoonotic transmission or accidental spill-over through exponential proliferation are also possible. In addition, a new host like a human would lack previous immunity to the pathogen, which provides a compatible incapacitating host defense mechanism[38]. Above all, the increasing human-animal interactions due to deforestation, hunting, domestication, wet market, and wild animals as a food source in many countries further increases the chance of viruses to adapt to infect humans[39]. Five SARS-CoV-2 strains are currently prevalent[40-44] (Table 1).

Prognosis and pathophysiology: thwarting effects of SARS-CoV-2 on patients with existing liver diseases

Various factors govern the prognosis of this disease based upon pre-existing health conditions, comorbidity, age, course of treatment adopted, and the response to treatment. The overall fatality rate is above 2%. Like most pathogens, the liver serves as one of the preferred sites of proliferation spots for coronaviruses because it is a common gateway for the blood[45,46]. It has been observed in many studies that one-third of patients with COVID-19 develop liver dysfunction, which was more frequent among elderly, male patients[47]. A survey conducted on 4000 seriously ill COVID-19 patients confirmed an inflated mortality rate after 3 mo, causing death in 31% of the patients[48]. Autopsies and post-mortem biopsies of the liver revealed infection of cholangiocytes, hepatocytes, and endothelial cells with SARS-CoV-2, which led to severe liver damage. Mechanisms of injury include major hepatocyte ballooning, eosinophilic action creating a cytokine storm, hypoxia, and ischemia leading to liver necrosis (Figure 2)[49-51].

Hepatic fat accumulation indicating microvesicular steatosis is a clear consequence of SARS-CoV-2 [52]. Additionally, lobular inflammation and fibrosis cause severe liver cirrhosis. Cirrhosis further reduces blood flow through the liver, thereby increasing blood pressure in the hepatic vein, which is



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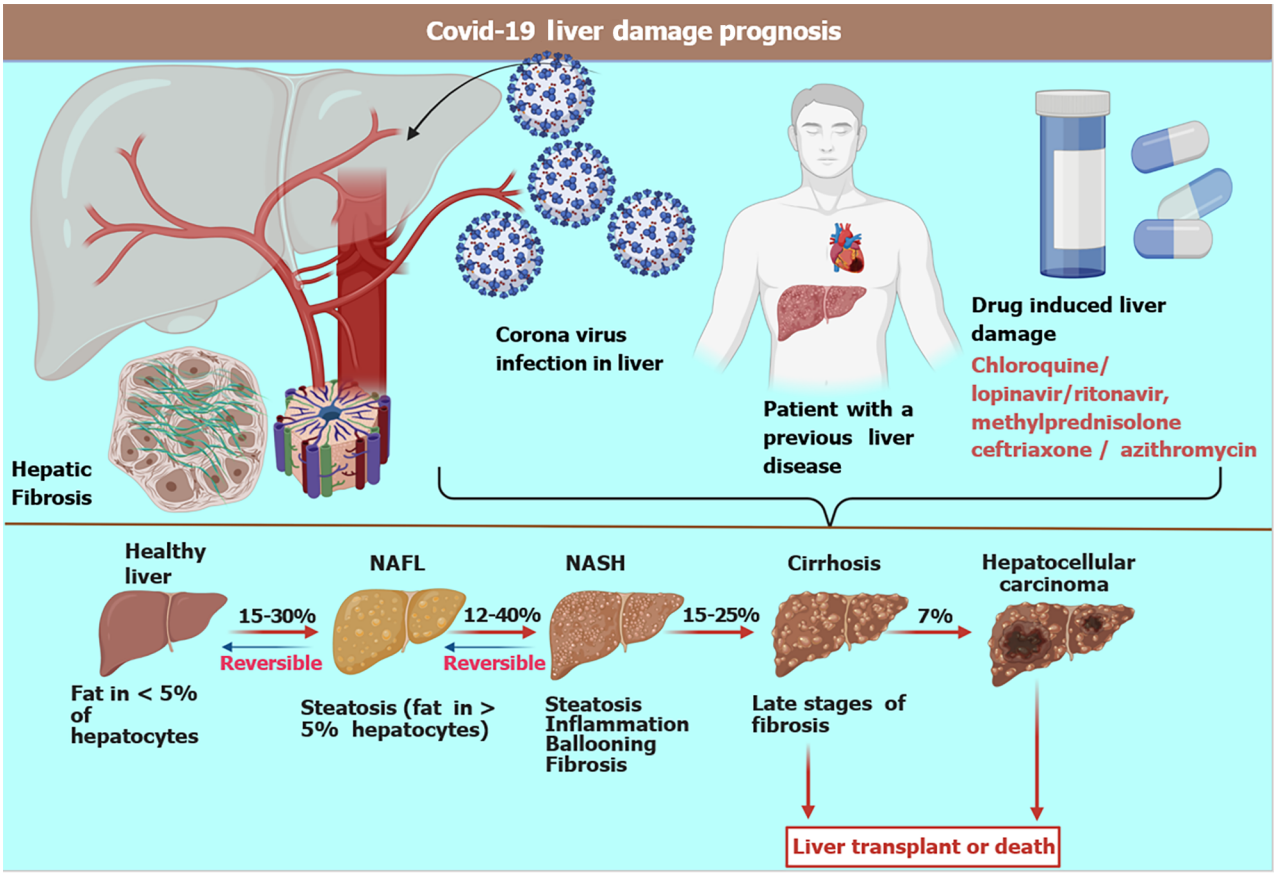
Figure 1 The origin and ancestral relationship of severe acute respiratory syndrome coronavirus 2 with other related viruses. Whole genome sequencing revealed that severe acute respiratory syndrome coronavirus 2 virus was most closely related to the CoV RaTG13 virus. The variant strains of coronavirus and their biogeographical distribution are illustrated. MERS-CoV: Middle East respiratory syndrome coronavirus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; R. affinis RaTG13: SARS-like coronaviruses: Bat-SLCoVZX.

supplied by the intestines and spleen. Once SARS-CoV-2 enters the human body, major histocompatibility complexes ensure the release of proinflammatory (acquired and innate)[53-55]. SARS-CoV-2 binds to the ACE2 receptor to enter the target cells[56]. These receptors are typically expressed on the bile duct epithelial cells, sinusoidal and capillary endothelial cells, and hepatocytes[57-59]. The viral antigen epitopes are recognized by antigen-presenting cells (macrophages and dendritic cells). They are presented to CD4+ T helper cells, lymphocytes, and natural killer cells, which activate B cells and CD8+ cytotoxic T cells[60-63].

Several patients with COVID-19 have abnormal biochemistry, displaying fluctuating levels of vital enzymes and biomolecules concerning hepatobiliary manifestations[64]. Patients with COVID-19 have decreased hepatic functions, typically displayed as increased liver enzymes and alanine transaminase and aspartate transaminase levels[5,65-67]. Several case studies observed these findings in more than 50% of critically ill COVID-19 patients[68]. Liver injury, chronic congestion, and nodular proliferation in patients with COVID-19 may occur, with more than 70% of patients developing steatosis[69,70]. The pathogenesis of hepatic damage in patients with COVID-19 is multifactorial, including pre-existing hepatic disease, hypoxia, ACE2 aided viral invasion and damage, ischemia, and drug-induced liver injury[71,72] (Figure 3).

MOLECULAR FEATURES: HEPATOTROPISM OR HEPATIC FRAILTY

The specificity of the virus to preferably invade hepatocytes is considered hepatotropism[65,73-75]. According to current research, SARS-CoV-2 does not specifically display hepatotropism but shows a preference for hepatocytes in patients with a pre-existing liver disease or a compromised immune system[76,77]. Patients with an existing liver disease display severe and prolonged symptoms of SARS-CoV-2 because the immune system dysfunction displays a more pronounced effect[45]. A meta-analysis of 90000 patients with COVID-19 pertaining to 40 case studies in the United States and China provided strong evidence for hepatic deterioration[78]. Hepatic frailty makes liver cells more susceptible and sensitive to COVID-19[79,80]. COVID-19 and comorbidities of hepatic diseases is a global phenomenon

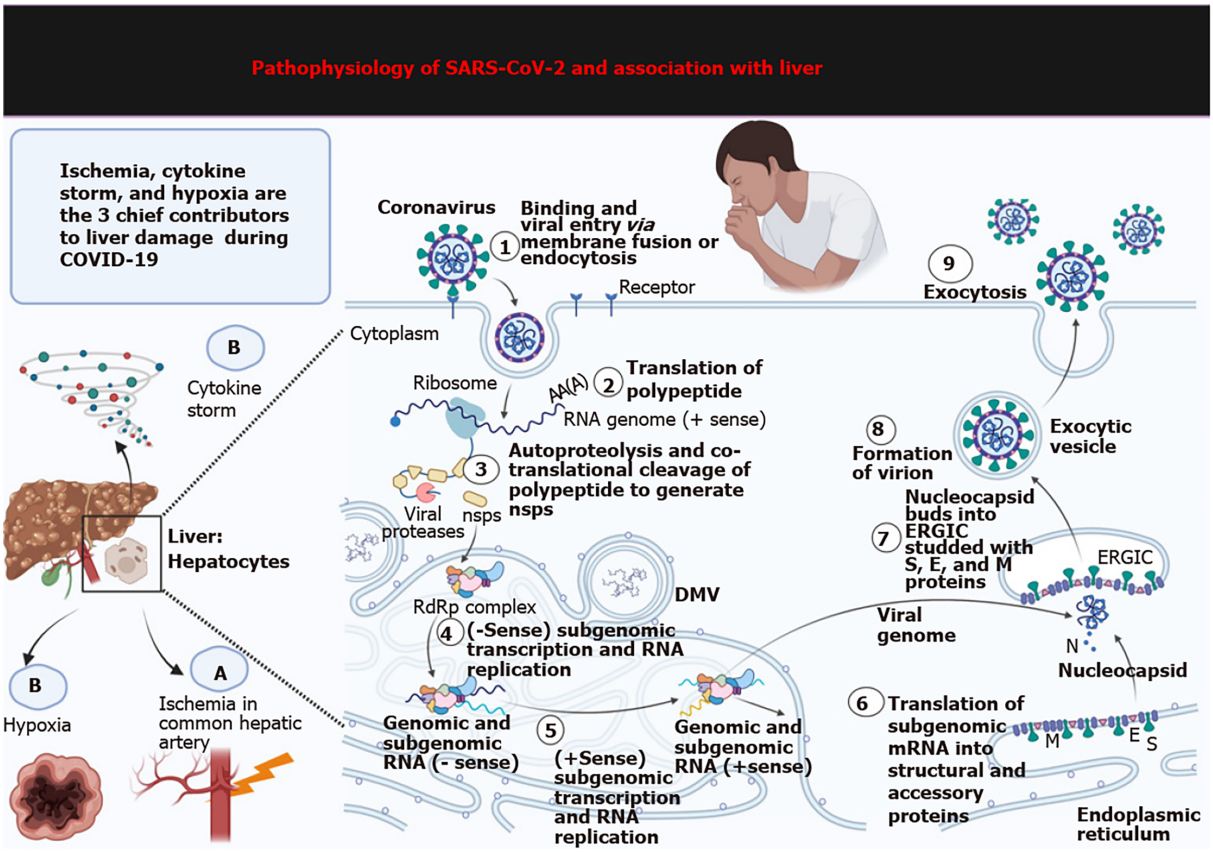


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Figure 2 The schematic representation of the effects of severe acute respiratory syndrome coronavirus 2 on the liver. A patient with a pre-existing liver disorder exhibits higher morbidity than patients with normal liver. Drugs may cause liver damage in some patients, making treatments ineffective. The stages of disease progression are implicated as steatosis (deposition of fat), causing a nonalcoholic fatty liver. The liver deteriorates, indicated by inflammation, ballooning of hepatocytes, and fibrosis. Severe liver cirrhosis is the next stage, which is irreversible. The final stage is hepatic cancer. COVID-19: Coronavirus disease 2019; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis.

[80,81].

The invasion of SARS-CoV-2 virus in the human body, its genomic single-stranded RNAs, and the replicative double-stranded RNAs are sensed by cytosolic RNA sensors. These are then identified and bound to NOD-like receptors, endosomal toll-like receptors, melanoma differentiation-associated gene 5, and retinoic acid-inducible gene-I-like receptors[82]. These receptors then stimulate the next set of effectors molecules downstream interferon (IFN) regulatory factor 3/7 (IRF3/7); activator protein-1; and nuclear factor-κB (NF-κB). The next step encompasses the synthesis of pro-inflammatory cytokines, namely, interleukin (IL)-2, IL-10, IL-6, IL-8; and IFN-I, by activating their transcription. These IFN-I molecules are thus supposed to be the first line of defense to combat and clear the viral particles from the body; these thus induce signal transducer molecules the Janus kinase 1 (JAK1)/tyrosine kinase 2 and transcription 1/2 (STAT1/2), turning on the JAK1/TYK2-STAT1/2 pathway. This generates STAT1/2/IRF9 complex that additionally triggers the transcription of IFN-stimulated genes. Thus, a cascade of events leads to the massive synthesis of antiviral chemicals: Procalcitonin; IL-6, CCL-5, IL-1, IFN-alpha, CXCL10, and CXCL-8, C-reactive protein[83]. Many studies have hinted at the unconventional triggering of certain supplementary systemic inflammatory responses leading to uncontrolled immune responses signaled by a storm of cytokines produced due to the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways[82]. This is commonly known as systemic inflammatory response syndrome, where a horde of immune cells (B cells, T-cells, natural killer cells, dendritic cells, neutrophils, and macrophages) bring about a cumulative and exaggerated response[84]. Apoptosis and cell death remain the culminating stage regulated by the MAPK pathway. Pyroptosis is a specialized mechanism induced by coronaviruses to prevent viral spread leading to an inflammatory caspase-1-dependent cell death in patients in response to rapid viral replication within infected cells[85]. In this activation process, the virus secures its persistence through the PI3 kinase/Akt pathway[86].



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Figure 3 Molecular mechanisms of liver damage during coronavirus disease 2019 infection. Hypoxia, ischemia, and cytokine storm significantly contribute to liver damage in coronavirus disease 2019-related comorbidities. This figure depicts how severe acute respiratory syndrome coronavirus 2 exponentially proliferates after entering hepatocytes. The steps occur: (1) Binding and viral entry *via* membrane fusion or endocytosis; (2) Translation of polypeptide; (3) Autoproteolysis and cotranslational polypeptide cleavage to generate non-structural proteins (nsps); (4) Sense subgenomic transcription and RNA replication; (5) + Sense subgenomic transcription and RNA replication; (6) Translation of subgenomic mRNA into structural and accessory proteins; (7) Nucleocapsid buds into ER-Golgi intermediate compartment studded with spike, envelope, and membrane proteins; (8) Formation of virion; and (9) Exocytosis. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; ERGIC: ER-Golgi intermediate compartment; DMV: Double-membrane vesicle.

PHARMACOLOGIC THERAPIES SPECIFIC TO CORONA-ASSOCIATED HEPATIC MORBIDITIES

Scientists worldwide could conceive fairly early the devastating effects of SARS-CoV-2, and social distancing was the only way out since impending outcomes were far from the view[87,88]. As an opportunist virus, it intimidated the whole world, shutting down everyday life and hampering the economy and health worldwide. Though we have successfully tamed this dangerous and feral pathogen, the efficacy of existing vaccines and drug therapies in preventing SARS-CoV-2 variants is still a matter of concern[87-89]. Vaccines were tracked on the plan to target spike proteins of the SARS-CoV-2, which the virus variants inventively cons[90-92]. Several novel vaccines, as well as drugs, have ardently helped in tackling these viruses. Since the virus shows high transmissibility and the future modulation in these viruses is erratic and unforeseen[90,92,93], prevention and management strategies should entail a multi-omic, closed-loop follow-up and holistic approach comprising scientists, government authorities, clinicians, pharmacists, and as the general public. Thus, prevention and management, including pharmacologic therapies against COVID-19, have been worked out under different approaches certified under Emergency Use Authorization[94-96]. Several therapeutic strategies are followed depending on the patient's condition as diagnosed by the clinician[97,98].

Immuno-modulators and anti-inflammatory agents

More appropriate to provide in the later stage of COVID-19 infection. Clinical trials conducted on 113 COVID-19 patients critically suffering from this disease, with both Baricitinib (inhibitor of Janus kinase) and Anakinra (IL-1 antagonists on 52 COVID-19 patients), have shown promising results in the case of COVID-19 patients facing hyperinflammation (cytokine storm). These offer a dual inhibitory effect by preventing both entries of SARS-CoV-2 and preventing an exaggerated cytokine response[99-102]. Such trials have attested to the efficacy of critical-stage COVID-19 patients, especially those with hepatic

Table 2 Summary of the effective vaccines developed so far against coronavirus disease 2019

Sr. No.	Name of vaccine	FDA approval	Type of vaccine	Manufactured by	Efficacy	No. of doses	Safety profile	Ref.
1	NVX-CoV2373 vaccine	December 20, 2021	Recombinant SARS-CoV-2 nanoparticle	Novavax	92.6%	2	Safe till date	[105]
2	BNT162b2 vaccine	FDA issued a EUA on December 11, 2020	mRNA-based	BioNTech/Pfizer	95%	2	Safe till date	[106]
3	mRNA-1273 vaccine	FDA issued a EUA on December 18, 2020	mRNA-1273 based	Moderna	94.1%	2	Safe till date	[107]
4	ChAdOx1 nCoV-19 vaccine	Not yet received a EUA or approval from the FDA	Recombinant spike protein vaccine	Serum institute of India, private limited	70.4%	2	Vaccine-induced immune thrombotic thrombocytopenia	[108]
5	Ad26.COV2	EUA by the FDA on February 27, 2021	Recombinant vaccine	Janssen-Cilag International, NV Belgium	73.1%	1	Vaccine-induced immune thrombotic thrombocytopenia	[109]
6	Covaxin	EUA for adults	Whole inactivated virus-based	Bharat Biotech in collaboration with ICMR and NIV, India	64%	2	Safe till date	[110]
7	Sputnik V	EUA qualified	Human adenovirus vector	Russian direct investment fund	97.2%	2	Safe till date	[111]
8	CoronaVac	FDA issued under EUA	Inactivated virus alum-adjuvanted candidate vaccine	Sinovac biotech, China	51% against symptomatic SARS-CoV-2 infection, 100% against severe COVID-19	2	Safe till date	[112]

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; FDA: Food and Drug Administration; EUA: Emergency Use Authorization; ICMR: Indian Council of Medical Research; NIV: National Institute of Virology.

complaints[103,104]. Table 2 shows the currently used effective vaccines developed and successfully reduced morbidity and mortality across the world[105-112].

Antiviral drugs

These are more suitable during the early phase of Corona infection. Molnupiravir, a very effective drug that reduces both morbidity and mortality; paxlovid: Reduced 89% mortality (trial conducted on 1219 patients); remdesivir, hydroxychloroquine, lopinavir/ritonavir, ivermectin, and chloroquine are all Food and Drug Administration approved, but show little or no effect over Coronavirus; it is even not effective against corona variants[113,114]. Therefore, they are not recommended in case of patients with hepatic trouble during COVID-19. Also, some of these drugs (lopinavir/ritonavir, mitifovir, and tocilizumab) are not recommended and prescribed to patients with pre-existing liver diseases as they are known to cause cholestatic toxicity and hepatic injury[115-117].

Neutralizing antibodies against SARS-CoV-2

Antibodies naturally produced by the body of recovering patients or stimulated through vaccination can block the attachment and hence the entry of an enveloped viral pathogen inside the cell, conferring lifelong immunity[118-121]. Convalescent plasma transfusion therapy with a high anti-SARS-CoV-2 immunoglobulin G (IgG) titer effectively lowered the mortality of critical COVID-19 patients[122-124]. The bamlanivimab and etesevimab antibody combination has been found to be super effective in COVID-19 patients with 87% lower death rate[125]. Another antibody cocktail, REGN-COV2, constitutes a group of two IgG1 antibodies (casirivimab and imdevimab) that target the receptor binding domain of SARS-CoV-2 and thereby reduce both morbidity and mortality of COVID-19 patients by 70%[126].

CONCLUSION

Extraordinary efforts have reaped fruits. As per the World Health Organization report on COVID-19 Vaccine Implementation Analysis & Insights, 63.4% of the World's population today stands vaccinated against COVID-19 in September 2022 (https://www.who.int/publications/m/item/covid-19-vaccine-implementation-analysis-insights-2-september-2022), and India alone proudly puts up 68% of its population in the list of a fully vaccinated cluster. This was made possible due to the untiring efforts of clinicians and researchers braced and heavily funded by the government and private agencies to curb this callous pandemic. It is anticipated that once 100% global vaccination is achieved, the virus will no longer be felonious. However, there are reasons to negate this notion. One explanation is that despite marshy governmental efforts, many people are vaccine-hesitant for inexplicable motives which may hamper virus block[127,128]. Even if this temper is somehow overcome, the dynamics of remodelling human immunity to ongoing viral mutations and evolution is worth consideration. The co-evolution may equip the virus with new immune strategies to escape the human immune defense mechanism and maintain its virulence. According to the United States Centres for Disease Control and Prevention, viruses with new mutations are specifically a matter of concern and shall not be considered lightly[128]. Lifestyle, assess to wet markets, climate change, and increased animal-human interactions offer preferred gateways and richer niches to these evolving viruses[129]. The armchair experts in virology, immunology, and genetics contribute substantially to future mitigation strategies. It can no longer be one bug, one drug approach. Herd immunity is expectantly looked upon but is short-term and modulates the virus with new attacking feats. What is good to know is that though the future is unseen, this COVID-19 pandemic has taught us valuable lessons and equipped health agencies, clinical experts, and the general public to face the subsequent pandemic terror.

FOOTNOTES

Author contributions: Khullar N and Bhatti JS contributed equally, performed the majority of the writing, and prepared the figures and tables; Singh S and Thukral B performed data accusation and writing; Reddy PH provided input on writing the paper; Bhatti GK designed the outline and coordinated the writing of the paper; and all authors have read and approved the final manuscript.

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REVIEW

Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives

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Abstract

Experimental evidence supports the fact that changes in the bowel microflora due to environmental or dietary factors have been investigated as implicating factors in the etiopathogenesis of inflammatory bowel disease (IBD). The amassing knowledge that the inhabited microbiome regulates the gut physiology and immune functions in IBD, has led researchers to explore the effectiveness of prebiotics, probiotics, and synbiotics in treating IBD. This therapeutic approach focuses on restoring the dynamic balance between the microflora and host defense mechanisms in the intestinal mucosa to prevent the onset and persistence of intestinal inflammation. Numerous microbial strains and carbohydrate blends, along with their combinations have been examined in experimental colitis models and clinical trials, and the results indicated that it can be an attractive therapeutic strategy for the suppression of inflammation, remission induction, and relapse prevention in IBD with minimal side effects. Several mechanisms of action of probiotics (for *e.g.*, *Lactobacillus* species, and *Bifidobacterium* species) have been reported such as suppression of pathogen growth by releasing certain antimicrobial mediators (lactic and hydrogen peroxide, acetic acid, and bacteriocins), immunomodulation and initiation of an immune response, enhancement of barrier activity, and suppression of human T-cell proliferation. Prebiotics such as lactulose, lactosucrose, oligofructose, and inulin have been found to induce the growth of certain types of host microflora, resulting in an enriched enteric function. These non-digestible food dietary components have been reported to exert anti-inflammatory effects by inhibiting the expression of tumor necrosis factor-α-related cytokines while augmenting interleukin-10 levels. Although pro- and prebiotics has established their efficacy in healthy subjects, a better understanding of the luminal ecosystem is required to determine which specific bacterial strain or combination of probiotics and prebiotics would prove to be the

ideal treatment for IBD. Clinical trials, however, have given some conflicting results, requiring the necessity to cite the more profound clinical effect of these treatments on IBD remission and prevention. The purpose of this review article is to provide the most comprehensive and updated review on the utility of prebiotics, probiotics, and synbiotics in the management of active Crohn's disease and ulcerative colitis/pouchitis.

Key Words: Ulcerative colitis; Crohn's disease; Pouchitis; Dysbiosis; Microbiota; Inflammation

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Core Tip: Current treatments for inflammatory bowel disease (IBD), such as corticosteroids and immunosuppressants, have potential adverse effects, and a significant proportion of patients dependent on these treatments are exposed to these associated long-term side effects. The discovery of novel and efficacious therapeutic strategies is a worldwide goal of IBD research, and probiotics, prebiotics, and synbiotics can offer viable solutions. These products offer a novel strategy to deliver beneficial components into the gut and emerge as promising new treatments for IBD, as intestinal dysbiosis has been reported as a major cause of his IBD. The review highlights the current state and action mechanism of these microbial therapies along with various studies that have reported their effectiveness in restoring balance in the gastrointestinal microbiota and thus eventually reducing intestinal inflammation.

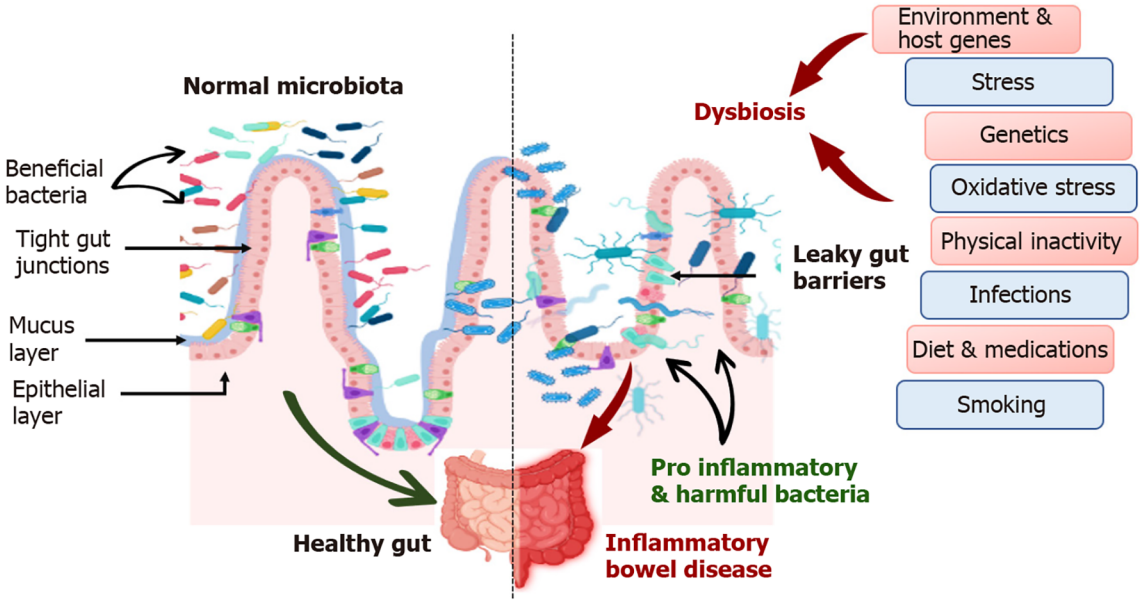
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INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic disease resulting from a debilitating immunological response of the body to the host's gastrointestinal (GI) microflora (mainly colon and duodenum). IBD classified as Crohn's disease (CD) and ulcerative disease (UC), was previously anticipated to be triggered by adaptive immune responses, however, the recent research findings suggest the prominent role of the innate immune system in instigating an imbalance and disparity between the beneficial microbiome and commensal microflora harboring in the human gut. This imbalance, known as dysbiosis (Figure 1), leads to an aggravated inflammatory response, causing IBD (Figure 2). Alterations in the microbiome affect host homeostatic systems and interactions with luminal stimuli, which can ultimately lead to uncontrolled inflammation in the intestinal mucosa, leading to IBD (Figure 3). This suggests that the human gut microbiome is beginning to be recognized for its important role and potential therapeutic solution for IBD. A better comprehensive understanding of the synergy between host genetics, external environmental factors and gut microbiome has opened new paradigms for seeking alternative effective therapies[1].

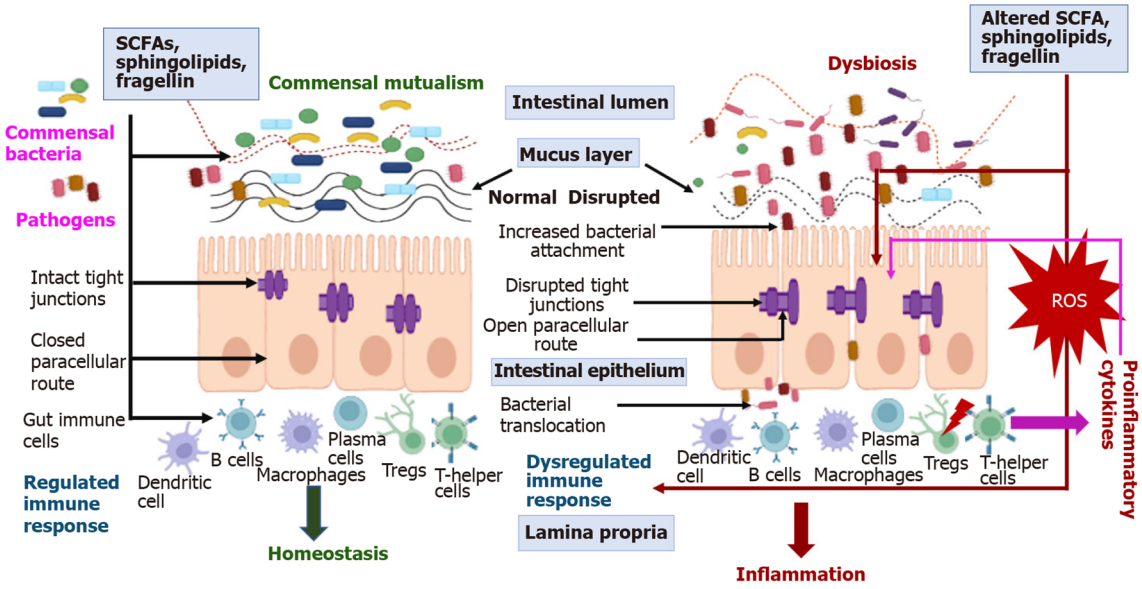
PROBIOTICS, PREBIOTICS, AND SYNBIOTICS AS THERAPEUTIC STRATEGY

Standard clinical treatment of IBD consists of agents that modulate the inflammatory pattern of the GI tract (GIT), including mesalamine, azathioprine, anti-tumor necrosis factors (TNFs), and glucocorticoids. However, these drugs often appear to have serious side effects, and some patients require higher doses throughout the course of treatment. A significant proportion of patients with IBD either initially do not respond to treatment or lose response over time. Gut microbiota modulation has emerged as an attractive new therapeutic approach for IBD, and gut microbiota-targeted/based therapies have been intensively investigated with varying degrees of success. Although the exact etiology of IBD remains unknown, the critical role of the gut microbiota in the development and persistence of IBD highlights the importance of microbiota-host interactions in health and disease. Recent advances in assessing the therapeutic potential of microbiota in the treatment of IBD support the reconstitution of microbial resident populations by administration of appropriate microbes. The gut microbiota influences the host by modulating physiological, pathophysiological, and immunological processes. Experimental animal studies along with clinical data have confirmed the influence of the gut microbiome in ameliorating inflammation, highlighting its potential as a therapeutic strategy for treating inflammatory diseases. Numerous therapeutic strategies have been developed to modify and remodel the gut microbiome for



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Figure 1 Comparison between normal microbiome and dysbiosis.



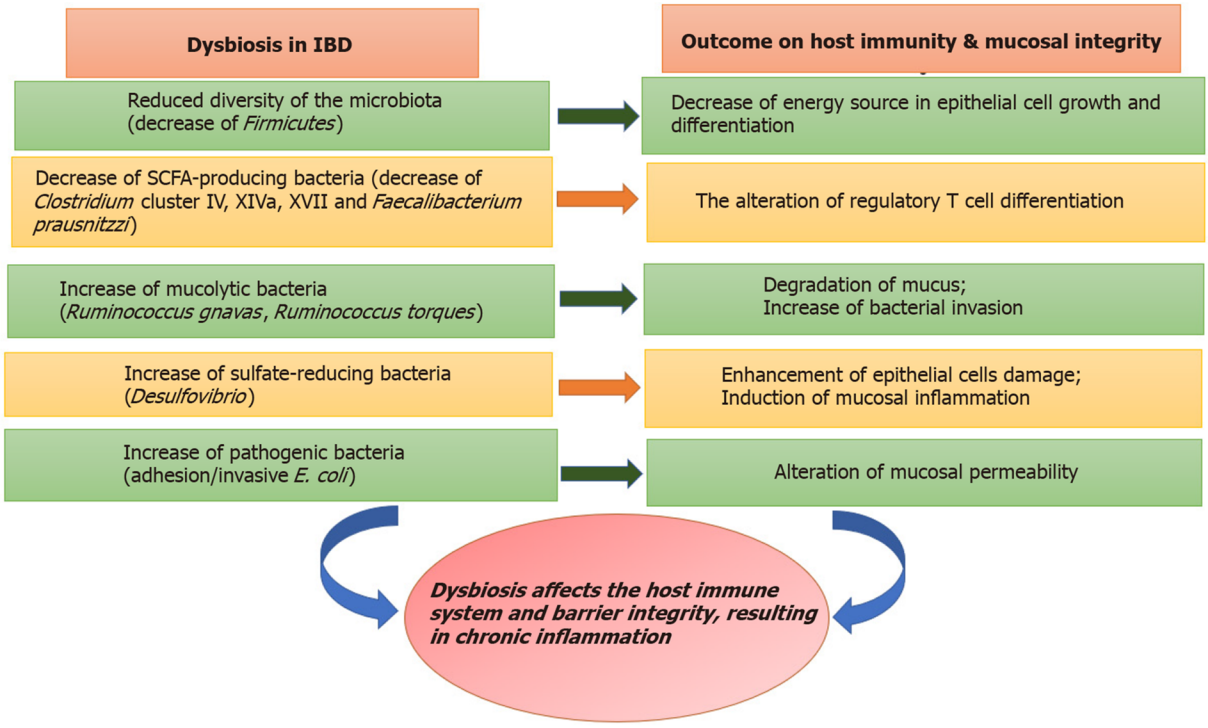
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Figure 2 Role of dysbiosis in inflammatory bowel disease pathophysiology. SCFA: Short chain fatty acids; Tregs: Regulatory T cells; ROS: Reactive oxygen species.

the treatment of other GI diseases, including IBD. Prebiotics, probiotics, synbiotics, and fecal microbiota transplantation (FMT) are currently considered to be the most common treatments[2].

PROBIOTICS

Probiotics are specific live microorganisms, when consumed in appropriate amount, are beneficial to the health of the host. Probiotic therapy involves the targeted introduction of beneficial microorganisms into the intestinal flora. This causes many beneficial bacteria to compete for nutrients and starve harmful bacteria. Probiotics participate in many positive health-promoting activities in human physiology, including the maintenance of a healthy gut. The most common strains currently available as probiotics and possessing beneficial health effects are *Enterococcus faecium, Bifidobacterium, Bacillus, Saccharomyces boulardii* (*S. boulardii*), *Lactobacillus strains,* and *Pediococcus*. Molecular mechanisms for the



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Figure 3 Dysbiosis in inflammatory bowel disease and its pathological outcomes. IBD: Inflammatory bowel disease; SCFA: Short chain fatty acids.

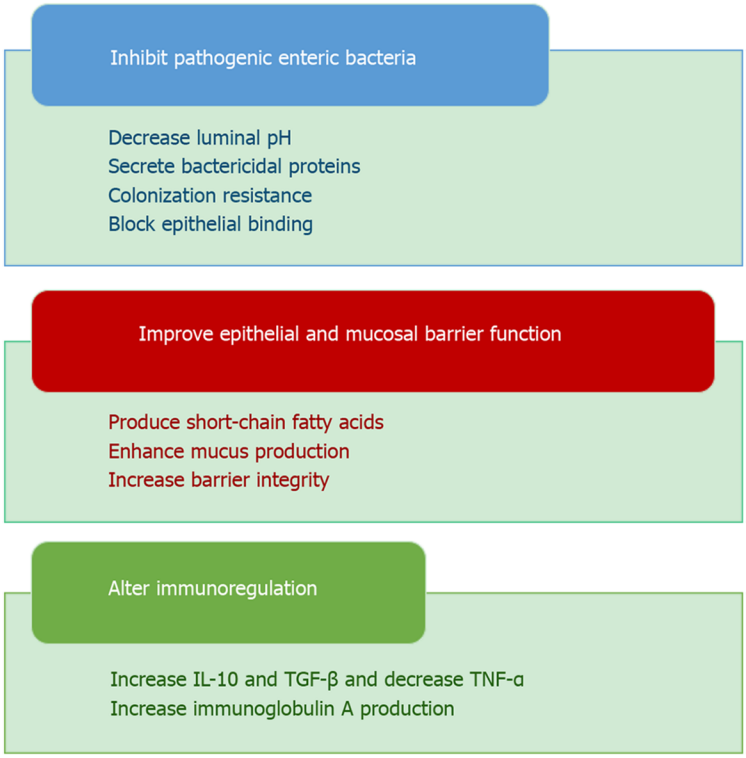
beneficial effects of these probiotics include (Figure 4): (1) Production of butyrate, immunoglobulin A (IgA), and short-chain fatty acid (SCFA) formation and stimulatory signaling proteins; (2) Reduced secretion of pro-inflammatory cytokines; (3) Increased mucin-2 expression; (4) Increased autophagy; and (5) Augmented upregulation of defensins. Although probiotics have shown promise both preclinically and clinically, the theoretical risks have been explained in several case reports, clinical trial results, and experimental models. These include systemic infections, adverse metabolic activity, overstimulation of the immune system in susceptible individuals, gene transfer, and GI side effects[3].

PREBIOTICS

Prebiotics are non-digestible food ingredients that selectively stimulate the growth of beneficial bacteria or promote the activity of a limited number of health-promoting bacteria. However, prebiotics can also help to improve the existence and effectiveness of ingested PRO bacteria. According to the most recent definition, "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the GI microbiota that confers benefits upon host well-being and health". Now, the International Scientific Association of probiotics and Prebiotics has introduced a new definition of prebiotics as "a substrate which is selectively fermented by the gut microflora and bestows health benefits to the host". This new definition states that non-carbohydrate constituents are also considered prebiotics and their applications are not constrained to the GIT only[4].

SYNBIOTICS

Combinations of probiotics and prebiotics are viewed as promising new approaches and provide an opportunity to explore their potential and efficacy in human IBD. When probiotics and prebiotics are combined in a product to achieve synergistic actions, they are commonly referred to as synbiotics. Many examples have demonstrated that prebiotics appears to be more efficacious when used along with a probiotic as a part of the synbiotic combination. The term synbiotic refers to synergism where the prebiotic component is selectively favoured by the live probiotic organism. The synbiotic combination is intended to enhance the *in vivo* survival and activity of proven probiotics to promote or enhance the beneficial properties of both products. However, recently the term 'synbiotics' has been re-defined as preparations favoring synergism, where the probiotics metabolize the complemented prebiotics to induce specific rebalancing of the dysbiotic gut and host health. Synergistic probiotics and prebiotics stimulate selective microbial growth or activate specific metabolism *via* gut flora. The presence of the



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Figure 4 Various actions of probiotics. IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha.

readily fermentable substrate should enhance the survival of the probiotic. The prebiotic component should also protect the probiotic from gastric acidity and proteolysis, possibly through steric hindrance and coating of the probiotic. Therefore, it is important to select specific substrate and microbial combinations in synbiotic products that can enhance beneficial effects compared to products containing probiotics or prebiotics alone[5].

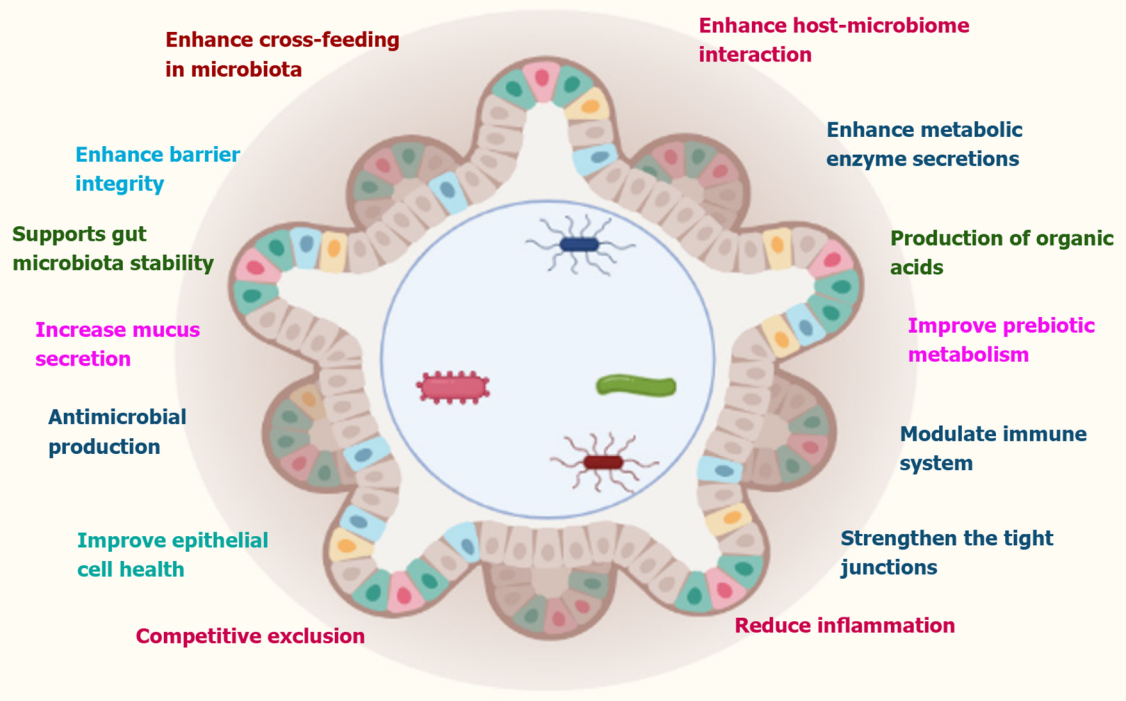
MECHANISM OF ACTION OF PROBIOTICS, PREBIOTICS, AND SYNBIOTICS

Probiotics

Probiotics, prebiotics, or synbiotics can achieve therapeutic effects in IBD through various mechanisms. The mechanisms of action of probiotics include competitive actions with commensal and pathogenic bacteria and effects on epithelial function and immune responses. By augmenting the production of SCFA, they can lower the pH of the intestinal environment, thereby inhibiting the growth of potentially pathogenic microorganisms. Some probiotics enhance the integrity of the mucosal barrier thereby normalizing intestinal permeability[6]. The effects of probiotics vary and depend on type and dose as well as on their interaction with the host in different ways. Some exhibit direct antibacterial action *via* the production of substances such as bacteriocins, hydroperoxides, lactic acid, and defensins. Others exhibit non-immunological action such as competing with pathogens for nutrients, increasing mucus production, changing intestinal pH, by promoting the formation of tight junctions (TJs), or enhancing tissue repair processes, thereby reducing intestinal mucosal permeability. Finally, probiotics can also modulate the immunological response (immunoglobulin production, pro-inflammatory cytokine production) by releasing cell wall fragments or DNA in the intestinal lumen (Figure 5). They also regulate the overactivation of the nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway, reduce the production and secretion of pro-inflammatory cytokines [such as interleukin (IL)-8, TNF-α, interferon gamma (IFN-γ)], and induce the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β[7].

Prebiotics

Prebiotics exert beneficial effects on IBD through multiple mechanisms of action. Primarily they accelerate the selective proliferation of native bacteria of the gut microbiota. These ingredients provide a better breeding space for beneficial microorganisms due to their loose structure and large surface area and at the same time inhibit the growth of pathogens. Secondly, they increase the production of SCFAs



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Figure 5 The mechanism of actions of probiotics in combating inflammatory bowel disease symptoms. IBD: Inflammatory bowel disease.

such as acetate, butyrate, and propionate. These SCFAs are formed during the fermentation of prebiotics and play a very vital role in the proper functioning of the intestine. They accelerate the regeneration and healing process of the intestinal epithelial cell; augment mucus production; maintain the correct pH in the intestine. They also inhibit the attachment of pathogenic microbes to enterocytes. Acetate is commonly used as a cellular fuel for building muscle and colonic tissue. Butyrate exhibit various beneficial effects on the host, such as improving metabolism, modulating the host's immune system, and promoting anti-inflammatory actions, therefore receives special attention[8].

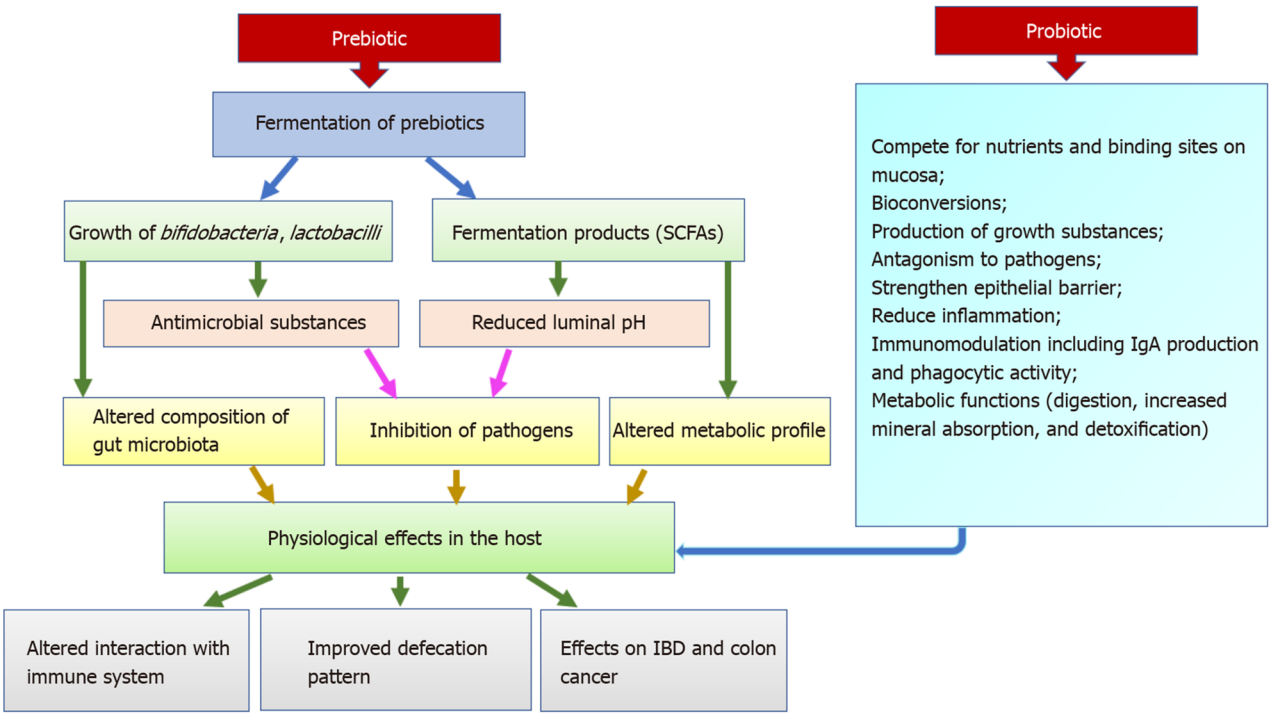
Therefore, prebiotic consumption has been shown to boost host immune function, reduce infection rates, enhance colonic integrity, and downregulate allergic reactions. However, these effects are not directly imposed by ingesting prebiotics. It was suggested that the benefits of prebiotics are achieved indirectly. Prebiotics improves the mucosal barrier *via* encouraging the probiotics growth that can upregulate epithelial defense mechanisms[6].

Synbiotics

In vitro studies specify that synbiotics exert primarily anti-inflammatory actions along with some antiproliferative activities. The literature on synbiotics is difficult to interpret, as it is often impossible to distinguish whether the desired therapeutic benefits are attributable to prebiotics, probiotics, or synergistic interactions between them. Various studies have provided robust preliminary evidence that synbiotic administration to IBD patients results in beneficial therapeutic effects. In one study, prebiotic Synergy 1 in combination with *Bifidobacterium longum* improved sigmoidoscopy scores and reduced b-defensins, TNF-α, and IL-1α in biopsy samples from UC patients. In another study, patients who received *Bifidobacterium longum* and prebiotic Synergy 1 [with Fructooligosaccharides (FOS)/inulin blend] combination revealed a significant histological improvement in comparison to the placebo group. Synbiotics significantly reduced the TNF-α expression and thereby reported the potential beneficial effect of synbiotics in the management of IBD (Figure 6). Combinations of synbiotics may exert beneficial impacts on the intestinal mucosa. Therefore, evaluating the role of synbiotics as an alternative form of IBD treatment should be considered[9].

PROBIOTICS IN IBD

Research on the use of probiotics in the treatment of IBD has been conducted since 1997. A 50% increase in probiotic use has been reported in IBD patients. This is due to the belief that probiotics are safe and beneficial as adjunctive therapy for IBD patients in both exacerbation and remission. Despite the moderately huge data reports on the use of probiotics in IBD, the possibility to draw firm conclusions is significantly limited. This may be due to the small number of patients in the study groups, large



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Figure 6 Mechanisms of action of probiotics, prebiotics, and synbiotics. SCFA: Short chain fatty acids; IBD: Inflammatory bowel disease.

differences in intervention types, or lack of standardization in study methods. There are also few published clinical studies on the effects of probiotics on inflammatory changes examined by GI endoscopy in IBD patients. However, the potential use of well-selected commensal microbial species with protective effects on the intestinal mucosa and modulation of immune responses offers hope for new treatment options for patients with IBD[9]. Various preclinical studies have been conducted to explore the efficiency of probiotics in the IBD are summarized in Table 1 and the clinical interventions are summarized in Table 2.

Effectiveness of probiotics in the animal models of colitis

Eradication of reactive oxygen species (ROS) by antioxidant enzymes such as catalase from the inflammatory site may efficiently restrain IBD pathogenesis. A genetically engineered probiotic *E. coli* Nissle 1917 (EcN), which acts by overexpressing the catalase and superoxide dismutase, was evaluated for the treatment of intestinal inflammation in a mouse IBD model induced by dextran sodium sulfate (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and oxazolone. The probiotic bioavailability in the GIT was increased by the application of chitosan and sodium alginate effective biofilms. It effectively relieved inflammation and repaired epithelial barriers in the colon and restored the expression of TJ-associated proteins. It also regulated the gut microbial flora and augmented the abundance of vital microbes that helped in the maintenance of intestinal homeostasis such as *Lachnospiraceae*_NK4A136 and *Odoribacter* [10].

With remarkable advances in genetic engineering, scientists have recently developed bacterial/probiotic strains that are genetically engineered to function as 'gut biosensors' that can help to detect inflammatory markers or 'resident cell factories' of therapeutic molecules that will act as biotherapeutic drugs to improve the drug delivery at mucosal surfaces[10].

Wang *et al*[11] showed that an engineered EcN discharging the immunoregulatory protein Sj16 isolated from the helminth *Schistosoma japonicum* alleviated the DSS-induced colitis in mice by modifying the gut microbiota. The immunoregulatory protein exhibited a protective effect against colitis through its action on the peroxisome proliferator-activated receptor-alpha (PPAR-α) receptor, reestablishing populations of the *Ruminococcaceae* family, thereby augmenting intestinal butyrate levels.

Zhang *et al*[12] established a constitutively expressing IL-35 *E. coli* as a novel oral delivery system with immunosuppressive actions, facilitated by regulatory T cells and B cells. The IL-35-producing *E. coli* demonstrated a decrease in the inflammatory response in a mice model of colitis by downregulating Th17 cells.

Lactobacillus paracasei has been genetically engineered with the human N-acylphosphatidylethanolamine-specific phospholipase D gene and, when potentiated with an ultra-low exogenous dose of exogenous palmitate, it selectively induced palmitoylethanolamide in the GIT. PEA exerted potent anti-inflammatory effects, and it had showed improvement in inflammation in animal models of colitis[13].

Table 1 Summary of preclinical studies of probiotics

Ref.	Model	Treatment	Dose and duration	Parameters analyzed	Conclusion
Yoo *et al* [26], 2022	DSS-induced colitis in male C57BL/6 mice	*Lactobacillus plantarum*, *Bifidobacterium longum*, and *Bifidobacterium bifidum*	1 × 10^9 CFU once daily for 5 d	Cytokines and corticosterone, colonic MPO activity, fecal LPS level	Suppressed colonic inflammation, and fatigue by the suppression of the IL-1β or IL-6 to IL-10 expression ratio and gut bacterial LPS production
Qin *et al* [27], 2022	DSS-induced colitis in mice	*Lactobacillus (Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Weissella cibaria)*	-	DAI, colon length, pathological score, cytokine secretion	Showed the potential to treat IBD
Khan *et al*[28], 2022	DSS-induced colitis in mice (C57BL/6)	*Lactobacillus plantarum*	-	Colitis indexes, IL-17A, IL-17F, IL-6, IL-22, and TNF-α and anti-inflammatory cytokines, *i.e.*, TGF-β, IL-10	Restored gut microbiota balance and modulated the resident gut microbiota and immune response
Fei *et al* [29], 2022	DSS-induced colitis in mice (C57/BL6)	*Ligilactobacillus salivarius* Li01 and RSV	Li01 (10^9 CFU/d) and RSV (1.5 g/kg/d)	IL-1β and IL-6, TGF-β and IL-17A	An improved synergistic anti-inflammatory effect from the RSV and Li01 combination treatment
Liu *et al* [30], 2021	DSS-induced colitis in mice (C57/BL6)	Goji juice fermented by *Lactobacillus plantarum*, *Lactobacillus reuteri* and *Streptococcus thermophilus*	20 mL/kg/d for 30 d	Pro-inflammatory cytokines and total superoxide dismutase in serum and colon, MPO and glutathione peroxidase	Probiotics-fermentation enhanced the anti-ulcerative colitis function of goji berry juice and modulated gut microbiota
Kim *et al* [31], 2020	DSS-induced colitis in male C57BL/6 mice	*Lactobacillus plantarum* CBT LP3 (KCTC 10782BP)	1 × 10^8 bacteria in 0.1 mL PBS once daily for 16 d	DAI, analysis of macrophages and T cell subsets gene expression and cytokine profiles	Effective anti-inflammatory effects, with increased induction of Treg and restoration of goblet cells, suppression of proinflammatory cytokines
Chen *et al*[32], 2020	DSS-induced colitis in C57BL/6J mice	*Bifidobacterium breve*, CCFM683	0.2 mL (10^{10} CFU/d CCFM683, once daily for 2 wk	Weight loss, stool consistency and fecal blood	Improved intestinal epithelial barriers, restored gut microbiota
Chen *et al*[33], 2020	DSS-induced colitis in C57BL/6 mice	*Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Enterococcus faecalis* with (quadruple probiotics, Pqua) or without (triple probiotics, P-tri) aerobic *Bacillus cereus*	*B. infantis*, *L. acidophilus*, and *E. faecalis* (1.5 × 10^9 CFU respectively) in 200 uL PBS and *B. cereus* (0.5 × 10^8 CFU) in 200 uL once daily for 45 d	Intestinal inflammation and functions of multiple barriers, including the mucus barrier, and epithelial barrier	Effective (Aerobe-contained Pqua was a powerful adjuvant therapy for chronic colitis, *via* restoring the intestinal microflora and recovering the multi-barriers in the inflamed gut)
Komaki *et al*[34], 2020	Mice	*Lactococcus lactis subsp. lactic JCM5805*	1 mg, 5 mg, 10 mg, 15 mg, or 20 mg, once daily for 1 wk	The survival rate, length, histopathological parameters of the colon, and concentrations of inflammatory cytokines in serum	Effective (high-dose administration deteriorates intestinal inflammation)
Silveira *et al*[35], 2020	C57BL/6 mice	*Lactobacillus bulgaricus*	1 × 10^9 CFU was diluted in 200 mL of PBS, 3 times per week for 12 wk	Intestinal inflammation, cytokines levels were determined from colon and/or tumor	Regulates the inflammatory response and prevents Colitis-associated cancer

DSS: Dextran sodium sulfate; CFU: Colony forming unit; LPS: Lipopolysaccharide; DAI: Disease activity Index; IBD: Inflammatory bowel disease; RSV: Resveratrol; MPO: Myeloperoxidase; IL: Interleukin; TNF-α: Tumor necrosis factor-α; TGF-β: Transforming growth factor-β; PBS: Phosphate buffer solution; Tregs: Regulatory T cells.

Liu *et al*[14] designed an engineered *Bifidobacterium longum R0175* (*B. longum*) that expresses the antioxidant enzyme manganese superoxide dismutase. The probiotic helped to improve colitis symptoms by attenuating the ROS-mediated oxidative stress and constraining endothelial cell activation. Following the treatment, attenuation in TNF-α, IL levels, as well as a complete improvement in the macroscopic and microscopic inflammatory markers was observed. Yet another study reported the beneficial effect of genetically modified *B. longum* that expresses αmelanocyte-stimulating hormone in a DSS model of colitis. The probiotic exhibited significant anti-inflammatory properties by suppressing the release of proinflammatory cytokines such as ILs, TNF-α, and NO, while increasing the anti-inflammatory cytokine (IL-10) release[15].

Feng *et al*[16] explored the ameliorating effects of pasteurized probiotic fermented milk on DSS-induced IBD in rats and found that intragastric gavage of milk prominently declined the disease activity index (DAI) scores and alleviated the colon tissue damage. The improvement was ascribed to the anti-inflammatory effect of the probiotic by decreasing TNF-α and IL-6 levels. The pasteurized probiotic fermented milk alleviated IBD by reducing the inflammatory response and restoring the gut microbiota.

Table 2 Summary of clinical studies of using probiotics in inflammatory bowel disease patients

Ref.	Treatment	Dose and duration	Parameters analyzed	Conclusion
Agraib *et al* [43], 2022	*Lactobacillus* and *Bifidobacterium* species	3×10^{10} probiotic capsules	Partial mayo score, CRP, IgA, IL-10, hemoglobin, hematocrit, and RBC levels	Significantly induced remission in UC patients
Ojetti *et al* [44], 2022	*Limosilactobacillus reuteri* ATCC PTA 4659	Two times a day for 10 d	Inflammatory markers CRP and calprotectin	Significantly reduced both blood and fecal inflammatory marker
Wu *et al* [45], 2021	Live *bacillus licheniformis* capsules and live combined *Bifidobacterium, lactobacillus,* and *enterococcus* capsules followed by Chamomile capsules	*Bacillus licheniformis* capsules two capsules, three times a day and live combined *Bifidobacterium, lactobacillus,* and *enterococcus* capsules two capsules twice a day. Probiotics consumed at a level of 10^7 CFU/mL	Leiden Index of Depression Sensitivity, Beck Depression Inventory questionnaires	Probiotics tend to improve cognitive reactivity to the sad mood in CD patients
Bamola *et al* [46], 2021	*Bacillus coagulans* Unique IS-2	2 billion-CFU/capsule twice in day 4 wk	Presence of beneficial gut bacteria, serum cytokines, symptoms of the disease	Showed beneficial effect when administered along with standard medical treatment
Waal M van der *et al* [47], 2019	Nine bacterial strains (*Bifidobacterium bifidum* W23; *Bifidobacterium lactis* W51; *Bifidobacterium lactis* W52; *Lactobacillus acidophilus* W22; *Lactobacillus casei* W56; *Lactobacillus paracasei* W20; *Lactobacillus plantarum* W62; *Lactobacillus salivarius* W24; *Lactobacillus lactis* W19)	7.5×10^9 CFU per 3 g in powder form, once daily for 6 wk	Quality of life from a patient perspective (semi-structured interviews)	Effective
Kamarli Altun *et al* [48], 2022	*Enterococcus faecium, Lactobacillus Plantarum, Streptococcus thermophilus, Bifidobacterium lactis, Lactobacillus acidophilus, Bifidobacterium long*, and FOS	Probiotic strains (3×10^9 CFU) and FOS (225 mg/ tablet), 1 tablet twice a day for 8 wk	Hemoglobin, leukocyte, neutrophil-to-lymphocyte ratio, sedimentation, and CRP, clinical and endoscopic activity indices	Effective
Sun *et al* [49], 2018	*Clostridium butyricum*	420 mg per capsule, 1.5×10^7 CFU/g, 3 capsules 3 times a day for 4 wk	Change from baseline in IBD symptoms, quality of life, stool consistency, and frequency	Effective
Matsuoka *et al*[50], 2018	*Bifidobacterium breve* strain Yakult and *Lactobacillus acidophilus*	10 billion bacteria of Bifidobacterium breve and 1 billion bacteria of *Lactobacillus acidophilus*, one pack per day for 48 wk	Clinical procedure (change in abdominal symptom score from baseline. Sutherland DAI subscore, change in abdominal symptom scores (passage of flatus and bloating), and intestinal microbiota	Less significant effect

CRP: C-reactive protein; UC: Ulcerative colitis; CD: Crohn's disease; CFU: Colony forming unit; FOS: Fructooligosaccharides; DAI: Disease activity index; IBD: Inflammatory bowel disease; IgA: Immunoglobulin A; RBC: Red blood cell; IL: Interleukin.

Javed *et al*[17] showed that *Bifidobacterium infantis* had beneficial effects in alleviating TNBS-induced colitis. Supplementation with *Bifidobacterium infantis* demonstrated significantly less damage to mucosal cyto-structures and reduced the colitis symptoms. This demonstrates the importance of probiotics in protecting the goblet cells and epithelial cell layers[17]. Based on another research group, *Bifidobacterium bifidum* supplementation significantly increased IL-10 levels and decreased IL-1β levels in colonic sections, confirming anti-inflammatory effects. These results seem to support the regulatory properties of *Bifidobacterium infantis* and *Bifidobacterium bifidum* to reduce inflammation and clinical signs of colitis [18].

In IBD, a decrease in *Firmicutes* (F) abundance and an increase in the *Bacteroidetes* (B) bacteria are found to be associated with the disease progression. Early administration of *L. reuteri* DSM 17938 to C57BL/6J mice improved the abundance of *F* and diminished the abundance of B comparatively, thereby altering gut microbial homeostasis[19].

Live and dead *L. plantarum* AN1 administered to an IBD mouse model *via* drinking water exhibited intestinal regulatory and anti-inflammatory properties. A combination of two diverse probiotics (*Bifidobacterium bifidum* WBIN03 and *L. plantarum* ZDY2013) diminished the UC in mice by altering the microbiota and reducing oxidative stress as well as inflammation. The combination upregulated antioxidant factors and downregulated TNF-α in UC mice. A probiotic blend augmented the frequency of *F* and reduced the frequency of B[20].

The co-administration of the *L. fermentum* KBL374 and KBL375 amended gut dysbiosis and ameliorated colitis by decreasing the pro-inflammatory cytokine levels and augmented anti-inflammatory cytokines. These probiotics mechanism of action includes balancing the F/B ratio, epithelial cell

barrier improvement and altering cytokine secretion[21].

Numerous data reports the utilization of multi-strain probiotic preparations. The VSL#3 is well known for its efficiency in IBD. VSL#3 is a commercial probiotic blend composed of 8 bacterial strains. Four lactobacillus strains (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrueckii subsp. bulgaricus*), three Bifidobacterium strains (*B. breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*), and a streptococcal strain (*Streptococcus salivarius subsp. thermophilus*). Several studies have demonstrated the effect of VSL#3 on DSS-induced colitis[22]. One study showed that VSL#3 (0.5 mL/d) reduced gut bacterial diversity associated with tissue injury. Conjugated linoleic acid was locally produced by VSL#3, which suppressed colitis by targeting myeloid cell PPARγ in the colon. Moreover, the anti-inflammatory effect remains the most important therapeutic mechanism of VSL#3 in DSS-induced rat colitis[23]. Both live and heat-killed VSL#3 decreased the expression of IL-23, IL-6, STAT3, and phosphorylated STAT3 (P-STAT3) in colonic tissue, thus reducing DSS-induced colitis in rats. The expression of inflammation-related mediators such as iNOS, NFκB was also inhibited by VSL#3[22].

Various experimental studies have concluded that VSL#3 normally acts on four components of the intestinal barrier: Biological, chemical, mechanical, and immune barriers. Regarding biological barriers, VSL#3 can increase the abundance of commensal gut bacteria and reduce the abundance of fungi. Regarding chemical barriers, VSL#3 can increase MUC2, MUC3, and MUC5AC gene expression and regulate mucus secretion. Regarding the mechanical barrier, VSL#3 can augment ZO-1 and occludin while attenuating claudin-2 to improve the function of TJs proteins. About the immune barrier, VSL#3 can inhibit the pro-inflammatory NFκB pathway while upregulating PPARα signalling[24].

A research group led by Hrdý *et al*[25] demonstrated that probiotic strains affect host cells in different ways. The mechanism of action of *Bifidobacterium animalis* species *lactis* B15764 and *Lactobacillus reuteri* Lr5454 were determined in mouse models of TNBS-induced colitis. Both strains exert beneficial effects on the host as expressed by body weight, gross indices of inflammation (Wallace score) and histopathological analysis (Ameho score), and lipocalin-2 levels in feces[25].

All of the above studies led to the inference that even a simplified preclinical model of colitis, which skips the genetic and external environmental influences, requires a broad and multidisciplinary approach.

Clinical study of probiotics among IBD patients

Several trials have reported the therapeutic action of the most common probiotic cocktail of proven efficacy, VSL#3 in adults with mild-to-moderate UC. In two clinical studies, VSL#3 was able to reduce DAI scores and significantly reduce clinical UC symptoms compared to the placebo. One study showed that 42.9% of patients treated with VSL#3 achieved remission in comparison to the placebo patients (15.7% remission only)[24]. Furthermore, VSL#3 and conventional drugs appear to have a synergistic effect. Although the mechanism is unknown, it is suggested that VSL#3 could enhance the anti-inflammatory effects of 5-aminosalicylic acid (5-ASA), inhibit free radical production, and suppress leukotriene and IL-1 production. A study also showed that combination therapy with VSL#3 and low-dose balsalazide was more efficacious than mesalazine or balsalazide alone in achieving remission of UC. Longer treatment with VSL#3 may result in greater improvement. Furthermore, in an open-label study, treatment with VSL#3 resulted in remission and improvement in 77% of patients with active mild-to-moderate disease UC. Two bacterial components *B. infantis* VSL#3 and *S. salivarius* subspecies *thermophilus* contributed a significant role in inducing remission by reaching the intestinal site of the disease. The effect of alkaline sphingomyelinase was also examined in 15 UC patients treated with VSL#3 for 5 wk and the outcome showed that VSL#3 upregulated mucosal alkaline sphingomyelinase activity and improved UC[25].

VSL#3 has also demonstrated valuable effects in children having IBD. A study showed that VSL#3 was operative in maintaining remission and reducing relapses in children with active UC. Apart from its role in maintaining remission, VSL#3 therapy also resulted in disease remission in children with mild to moderate acute UC as reported by another pilot study[36].

Jia *et al*[37] performed a meta-analysis of remission, relapse, and complication rates between EcN 1917 and mesalazine. The results demonstrated that there were no significant differences in the EcN1917 group or mesalazine-treated patients and were safe and well-tolerated. In summary, EcN 1917 has a comparable efficacy to mesalazine in terms of remission induction. This probiotic could be considered as an alternative for patients with IBD. Tamaki *et al*[38] reported that treatment with *Bifidobacterium longum* in patients with mild to moderate UC, prominently reduced DAI score and decreased rectal bleeding, as well as showed that they also achieved clinical remission. Treatment with probiotics and commonly used anti-inflammatory drugs together appears to be a more effective solution than treatment with probiotics alone. Palumbo *et al*[39] combined mesalazine with a probiotic mixture (*Lactobacillus salivarius, Lactobacillus acidophilus*, and *Bifidobacterium bifidum* strain). The combination showed beneficial effects among UC patients. The dual-treatment group demonstrated shorter recovery time, lower disease activity, and showed better endoscopic images. Another research group found that oral administration of *Bifidobacterium infantis* suppressed the CRP and TNF-α levels in both GI inflammatory diseases but did not specifically affect UC disease[40].

Also in one study, *S. boulardii* and VSL#3 in combination with conventional therapy in mild to moderate UC (involving 244 patients) showed no improvement in remission rates. However, only modest benefits were obtained in terms of reduction in disease activity[41]. EcN, *S. boulardii*, *B. breve,* and *Bifidobacterium bifidum* strains Yakult has demonstrated efficacy and safety similar to standard 5-ASA in maintaining remission in patients with mild to moderate UC based on histology, endoscopy, or quality of life. Presently, only four clinical trials have reported the use of *S. boulardii* as a probiotic therapy for IBD, with three reports of efficacy. Probiotic therapy using probiotic yeast such as *S. boulardii* could be a probable treatment for clinical trials, but the validation of reported efficacy in animal models requires multiple placebo trials[42].

An randomized controlled trial (RCT) evaluated the ability of VSL#3 to prevent the endoscopic recurrence of CD in humans after surgery. In this study, 10% of patients in the early VSL#3 group (VSL#3 administered throughout 365 d) had no severe lesions on day 90, but severe lesions developed on day 365 compared to the 26% of patients in the late VSL#3 group (administration of VSL#3 from day 90 to day 365). The finding suggested that VSL#3 exposure time was closely related to its therapeutic efficacy. However, DAI and IBD questionnaire (IBDQ) scores were similar in the two groups.

Few clinical studies have also shown that VSL#3 can prevent or maintain remission in chronic pouchitis. It was stated that after ileal pouch-anal anastomosis for UC, in the VSL#3 group 10% of patients had an onset of acute pouchitis when compared to the 40% of patients in placebo group. VSL#3-treated patients (17 patients, 85%) maintained antibiotic-induced pouchitis remission compared to placebo-treated patients (1 patient, 6%)[42].

PREBIOTICS IN IBD

Numerous studies have demonstrated the role of prebiotics on the intestinal flora and demonstrated that the use of prebiotics can enhance the metabolic function of the intestinal flora. Various studies reported that prebiotics diminishes the inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-12, TNF-α, IFN-γ, and improve the natural intestinal barrier by increasing the mucinous layer and TJs between epithelial cells. Their ability to decrease pathological bacteria in the gut and provide commensal bacteria with substrates capable of being metabolized into substances that contribute to the production and secretion of anti-inflammatory cytokines make them interesting candidates for various researchers working in IBD management. Surprisingly, more research has been done preclinically and a low number of significant prebiotic-associated human clinical trials are reported. The restricted research studies number is a foremost drawback and not adequately sufficient to support the use of prebiotics to treat IBD. Most prebiotics used in animal studies were polysaccharides derived from grapes, mushrooms such as *Ganoderma lucidum*, and herbs. In contrast, most human clinical trials described the usage of FOS for prebiotic treatment of IBD[34].

Prebiotic effectiveness in animal model of colitis

The use of prebiotics has shown promise in the management of colitis and is also widely used in animal models (Table 3). Various prebiotic preparations have been tested in animal models of colitis. The prebiotic effects of inulin have been studied in DSS-induced distal colitis in a rat model histologically resembling human UC. Daily administration of inulin by the oral route increases the number of natural *lactobacilli* in the lumen of the cecum and also lowers colonic pH. In rats with DSS-induced colitis, mucosal inflammation and histological injury scores are reduced by oral administration of inulin. Furthermore, inulin-fed rats showed a lower degree of mucosal damage and less severe crypt damage compared to controls. Treatment with orally administered inulin showed similarly beneficial effects, regardless of whether treatment was provided before or during the DSS exposure[51].

A nutritional combination of inulin and oligofructose at 5 g/kg body weight reduces intestinal inflammation in transgenic rats. An increase in *Bifidobacteria* and *Lactobacilli* was observed in the gut along with a decrease in pro-inflammatory cytokines and an increase in the growth factor-β that alters immune regulation. Taken together, these results suggest that combination therapy with different prebiotics may be more effective than monotherapy due to the fact that each drug has specific biological properties[52].

Pectic polysaccharides (PPS) are thought to be essential carbohydrates available to the gut microbiota, playing a dominant role in maintaining intestinal balance and are more potent than some conventional prebiotics. PPS can stimulate the growth of beneficial bacteria, such as *Lactobacillus*, *Bacteroides*, and *Bifidobacterium*, fully meeting the condition of a "stimulating probiotic". Some aberrant PPS have shown specific immunological capabilities compared to the PPS on the market. For example, sweet cherry PPS significantly induces the expression of NO and some immune proteins such as IL-6 and IL-10. Moreover, PPS from silver linden flowers enhances immunity in mice by inducing ROS and NO and suppressing iNOS. One study shows that PPS from *Gentiana crassicaulis* can enhance host immunity in terms of immune complement fixation[53].

Lactulose is reported to reduce inflammation and instigate the growth of lactic acid bacteria in IL-10 knockout mice while administration of inulin and germinated barley foodstuff (GBF) reduced DSS-

Table 3 Summary of preclinical studies of prebiotics

Ref.	Model	Type of treatment	Composition	Dose	Parameters analyzed	Conclusion
Cui *et al* [57], 2021	DSS-induced colitis in C57BL/6J mice	Polysaccharide from *Scutellaria baicalensis Georgi*	Mannose, ribose, rhamnose, glucuronic acid, glucose, xylose, arabinose, fucose	50 and 200 mg/kg once daily for 10 d	Body weight, loose stools, morbidity, hematochezia, and the DAI	Effective (attenuated body weight loss, reduced DAI, ameliorated colonic pathological damage, and decreased MPO activity)
Tolonen *et al*[58], 2022	DSS-induced colitis in male C57Bl/6 mice	Synthetic glycans	FOS, GOS, XOS, pullulan, and lactulose	1% (v/v) glycans (days 7-14)	Weight loss, scores of diarrhea, endoscopy, and colonic histology	Synthetic glycans increase survival, reduce weight loss, and improve clinical scores in mouse models of colitis
Qian *et al* [59], 2022	DSS-induced colitis in male C57Bl/6 mice	GOS, FOS along with FMT	FMT alone or combined with various ratios of GOS, and FOS	-	DAI scores, histology, protein or mRNA expression levels of FFAR3 and ZO-1, a tight junction protein	Treatment with FMT plus a prebiotic blend restores thestructure of the intestinal flora and increased the levels of acetic acid, butyric acid, FFAR3, and ZO-1
Liu *et al* [60], 2016	DSS-induced colitis in male C57BL/6 mice	Alpha D-glucan from marine fungus *Phoma herbarum* YS4108	Glucopyranose	40 mg/kg/d once daily for 1 wk	DAI scores, histology immunohistochemistry analysis, evaluation of SOD and MDA activities, and determinations of inflammatory cytokines	Effective (significantly increased butyrate, isovaleric acid levels, and prominent alterations on specific microbiota)
He *et al* [61], 2020	DSS-induced colitis in male C57BL/6 mice	Stachyose	Stachyose	1.5 g/kg/d for 28 d	Inflammatory cytokines including IL-6, IL-10, IL-17a, and TNF-α	Increased beneficial microbiota and bacterial diversity to alleviate acute colitis in mice
Kanwal *et al*[62], 2019		Dictyophora indusiate polysaccharide	Glucose 59.84%, mannose 23.55%, and galactose 12.95%	Low dose 10 mg/kg and (high dose 33 mg/kg once daily for 2 wk	Assessment of DAI, histological, analysis of goblet cells and mucus layer thickness, cytokines	Effective
Li *et al* [63], 2020	Male C57BL/6 mice	FMG or dealcoholized muscadine wine	FMG: Fructose 34.7% glucose 31%, sucrose 9.9%. DMW: Fructose, sucrose, and glucose not detected	FMG (7%, w/w) or DMW (5.5%, v/ w) for 3 wk	Bodyweight, stool consistency and bleeding, DAI, short-chain fatty acids in feces, and Mucin 2 and IgA in feces	Effective (reduced dysbiosis in the colon)
K-da *et al* [64], 2020	Male C57BL/6 mice	GFO	Monosaccharide composition in the GFO was D-galactose	100, 500, or 1000 mg/kg once daily for 2 wk	GI transit time, *ex vivo* propulsive motility, *in vitro* colonic smooth muscle contractility, the composition of colonic microbiota, and production of SCFAs	Effective (prevented and attenuated colitis symptoms and GI dysmotility, reducing populations of harmful bacteria and increasing SCFAs)

DSS: Dextran sodium sulfate; DAI: Disease activity Index; MPO: Myeloperoxidase; FOS: Fructooligosaccharides; GOS: Galactooligosaccharides; XOS: Xylooligosaccharides; FMT: Fecal microbiota transplantation; ZO-1: Zona occludens; SOD: Superoxide dismutase; MDA: Malondialdehyde; SCFAs: Short-chain fatty acids; GI: Gastrointestinal; IL: Interleukin; TNF: Tumor necrosis factor; FMG: Freeze-dried muscadine grapes; DMW: Dealcoholized muscadine wine; FFAR3: Free fatty acid receptor 3; GFO: *Gracilaria fisheri* oligosaccharides.

induced colitis in rats. It has been shown to increase the luminal concentration of SCFA, as well as increase the density of *Lactobacillus* and *Bifidobacterium*[54].

Mushrooms consist of diverse polysaccharides with prebiotic potential, including α-glucans, chitin, mannans, xylans, and galatians. Xie *et al*[55] stated that the prebiotics *Ganoderma lucidum* polysaccharide enhance SCFA-producing bacteria and augments SCFA production and suppresses DAI prominently. The study also described a decrease in infective microbiota such as *Shigella* and *Escherichia* in the rat model[55].

The effect of neoagarotetraose (NT), a hydrolytic product of agar by β-agarase, was evaluated in the DSS-induced murine model. The data show that NT intake improved intestinal integrity and inflammation scores. NT reversed the density of *Proteobacteria* from the DSS-induced increased levels[56].

Reduced clinical signs and increased MUC-3 expression were observed in rats that were nourished with goat's milk oligosaccharides as compared to the DSS-induced colitis rats. In trinitrobenzene sulfonates that provoke colitis rats, the colonic inflammation and necrotic lesions are also reduced by goat's milk oligosaccharides as compared with control rats[56].

Clinical study of prebiotic among IBD patients

Although there are few human studies using prebiotics, some new evidence suggests that the prebiotic treatment holds promise (Table 4). After colectomy for UC inulin showed a positive effect in the management of chronic pouchitis. A small, open-label study in 10 patients with active CD showed that the 21-d oral administration of 15 g of oligofructose and inulin significantly reduced the disease status. In UC patients, *Plantago ovata* (psyllium) outperformed placebo in reducing symptom severity and significantly increasing fecal concentrations of *Bifidobacteria*. Psyllium seeds formerly stimulated SCFA production, when tested as maintenance therapy for the UC patient in remission in an open randomized study. In this, patients received mesalamine (500 mg/d thrice daily for 1 year), psyllium seed alone (10 g twice daily), or a combination of both at the same doses. Remission were comparable in all groups, and a substantial augmentation in fecal butyrate concentration was detected after the administration of *Plantago ovata* seeds[65].

GBF consists of an extract rich in glutamine and hemicellulose. In small pilot and placebo-controlled studies, its use was evaluated in UC patients having mild to moderate disease severity. GBF significantly increased fecal levels of *Bifidobacteria* and reduced clinical and endoscopic activities at a dose of 25-30 mg/d. Comparable outcomes were described by 24-wk open-label study. A combination of 15 g/d oligofructoses and inulin was investigated on ten patients with active CD in a small open-label study. A substantial decrease in DAI accompanied by a substantial increase in mucosal *Bifidobacteria* was observed. Interestingly, prebiotics amplified colonic dendritic cells expressing IL-10, toll-like receptors-2, and 4, signifying the mechanism of the prebiotics on the mucosal innate immune response.

Wilson *et al*[66] explored the effects of prebiotic Galactooligosaccharides (GOS) supplementation on colonic inflammation in 17 patients with active UC. Patients reported improved stool consistency, decreased incidence and severity of loose stools, and decreased urgency of defecation after administration of GOS at 2.8 g/d for 6 wk. The proportion of *Bifidobacterium* and *Christensenelaceae* increased only in patients with low disease activity, suggesting that prebiotic effects may depend on disease activity. A controlled study is required to validate these observations to essentially determine if the GOS prebiotic is a useful adjunct therapy in active UC[66].

The effect of enteral inulin on ileal pouch function was assessed by examining epithelial gene expression, cell turnover, and mucosal morphology. The authors found that enteric supplementation with 24 g/d inulin increased butyrate production, reduced inflammation-related factors, decreased secondary bile acids, and significantly reduced endoscopic and histological DAI scores[67].

SYNBIOTICS IN IBD

Synbiotics not only improve the survival of beneficial microorganisms added to food and feed but are also used to stimulate the growth of certain natural bacterial strains present in the GIT. Given a large number of possible combinations, the application of synbiotics to modulate the human gut microbiota appears promising.

Effectiveness of synbiotic in animal models of colitis

Recently, several preclinical studies have shown that the use of probiotics and prebiotics as a synbiotic combination alleviates intestinal inflammation more than either probiotics or prebiotics alone (Table 5). The effects of formulated prebiotic mixtures, probiotic mixtures, and synbiotics were investigated in the colitis model induced by DSS in mice. Results in Synbiotic-treated colitis mice showed the preservation of colonic histological architecture and mucin production, upregulation of occludin expression, and diminished cell infiltration. A significant decrease in plasma IL-6 levels was observed after treatment. Treatment also modified gut microbiome, improved colonic integrity, upregulated anti-inflammatory cytokines, and suppressed inflammation markers, possibly through inhibition of IL-6/STAT3 signaling. In addition, synbiotic-treated mice displayed the highest levels of anti-inflammatory mediator IL-10 among the treatment groups in colitis mice. Among the treatments, synbiotics showed the most pronounced effect, indicating the highest potential for prevention and treatment of IBD[73].

Kangwan *et al*[74] demonstrated a protective effect of *L. pentosus* A14-6, CMY46 against DSS-induced intestinal inflammation. A14-6 and CMY46 are the novel strain of *L. pentosus* isolated from tea leaves (Miang) in Northern Thailand. The anti-inflammatory actions of *L. pentosus* CMY46 combined with GOS and *L. pentosus* A14-6 combined with XOS and were explored in C57BL/6 mice for 21 d. Synbiotics ameliorated DSS-induced colitis by preserving weight loss, reducing DAI, restoring colon length, and suppressing histopathological damage. Moreover, synbiotics enhanced intestinal barrier integrity and reduced colonic inflammation. Further, synbiotics possessed excellent anti-inflammatory and

Table 4 Summary of clinical studies of using prebiotics in inflammatory bowel disease patients

Ref.	Type of treatment	Dose	Parameters analyzed	Conclusion
Valcheva *et al*[68], 2022	β-fructans (oligofructose and inulin)	15 g/d for 6 mo	Mayo score. FCP, along with stool metabolites	Did not prevent symptomatic relapses in UC patients but reduced the severity of biochemical relapse and increased anti-inflammatory metabolites
Pietrzak *et al*[69], 2022	Sodium butyrate	150 mg sodium butyrate twice a day for 12-wk	DAI, FCP	As adjunctive therapy, it did not show efficacy in newly diagnosed children and adolescents with IBD
Vernero *et al*[70], 2020	Oral microencapsulated sodium butyrate (BLM)	Dose of two capsules/day for 12 mo (500 mg of BLM for each capsule)	DAI, FCP, CRP	BLM supplementation appears to be a valid add-on therapy to maintain remission in patients with UC
Valcheva *et al*[71], 2019	Oligofructose-enriched inulin	7.5 g (n = 12) or 15 g (n = 13) daily oral oligofructose-enriched inulin for 9 wk	Mayo score, endoscopic activity and FCP	15 g/d dose inulin-type fructans produced functional but not compositional shifts of the gut microbiota. Controlled studies for the use of β-fructans as an adjunct therapy in patients with active UC are required
Azpiroz *et al*[72], 2017	scFOS	5 g per sachet, twice daily for 4 wk	Rectal sensitivity, anxiety/depression, quality of life scores, and composition of fecal microbiota	Less significant (scFOS on rectal sensitivity may require higher doses and may depend on the subgroup)

FCP: Fecal calprotectin; DAI: Disease activity index; CRP: C-reactive protein; UC: Ulcerative colitis; scFOS: Short chain fructooligosaccharides.

immunomodulatory activities, as evidenced by decreased inflammatory mediator expressions of TNF-α, IL-1β, IL-6, and cyclooxygenase-2 (COX-2) in the colon. Symbiotic CMY46 in combination with GOS markedly increased IL-10 expression. These results suggest that synbiotics isolated from Mian are more effective than sulfasalazine[74].

The efficacy of *Bacillus amyloliquefaciens* enriched camel milk (BEY) was evaluated in TNBS-induced colitis mice models. Results showed that BEY treatment attenuated the proinflammatory cytokines (IL-1 β, IL-6, IL-8, and TNF-α) and myeloperoxidase levels. In addition, the protein markers such as phosphatase and tensin homolog, NFκB, COX-2, proliferation nuclear antigen, and occludin were substantially downregulated by BEY treatment. The BEY alleviated the colitis symptoms[75].

Bacillus coagulans FCYS01 spores in combination with chitooligosaccharides (COS) were evaluated for the possible ameliorating effects on DSS-induced colitis in mice. In comparison to the DSS group, the supplement significantly modulated the levels of CPR and cytokines IL-4, IL-6, IL-8, and IL-10. It significantly restored the TJ proteins and mucin protein expressions, thereby promoting the recovery of the intestinal barrier. Additionally, these dietary supplements improve SCFA production by modulating the composition of the gut microbiota and enhancing SCFA-producing bacteria. In conclusion, synbiotics mitigated the inflammatory status of the experimental UC model and showed better therapeutic efficacy than individual *B. coagulans* or COSs[76].

In another study, supplementation with synbiotics could substantially ameliorate the disease activity in DSS-induced acute colitis mice. The synbiotic significantly preserved the epithelial TJ proteins at colon, signifying the shielding of the intestinal barrier. The pro-inflammatory cytokines were reduced while augmentation of anti-inflammatory cytokines was mediated by the symbiotic treatment. The synbiotic used in the study was composed of 8 probiotic strains, including *Bifidobacterium animal*, *Lactobacillus paracasei*, *Bifidobacterium lactis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *B. breve*, *Lactobacillus fermentum*, and *Streptococcus thermophilus* along with FOS[77].

In another study, a synbiotic consisting of *Lactobacillus fermentum* HFY06 and arabinoxylan showed that the synbiotic can prevent and treat DSS-induced colitis. The results exhibited their synergistic effect by inhibiting the activation of the NFκB signaling pathway, upregulating the mRNA expression of NFκB inhibitor-α, downregulating mRNA expressions of NFκB-p65, inhibiting the cytokines TNF-α, inducible NOS, and COX-2, and exerted anti- colitis effects[78].

Synbiotic supplement with probiotic *Bacillus coagulans* spores and prebiotic green banana resistant starch ameliorated intestinal inflammation in the murine IBD model induced by DSS. A considerable efficacy of synbiotic supplementation was highlighted as it reduced the colitic manifestations and its severity. A significant anti-inflammatory effect was produced by suppressing abnormal immunological responses and colonic damage induced by DSS. Synbiotic accounted for about 29% increase in IL-10 levels and about 37% suppression of CPR along with about 40% IL-1β suppression compared to that of the DSS-control. The combination also improved SCFA production. The synbiotic supplementation amended the complete inflammatory condition *via* synergistic actions[79].

Table 5 Summary of preclinical studies of synbiotics

Ref.	Model	Probiotics	Prebiotics	Dose	Parameters analyzed	Conclusion
Xue *et al* [80], 2023	DSS-induced colitis in mice	*Lactobacillus plantarum* LP90	Soluble dietary fiber obtained from *Lentinula edodes* by products	1×10^9 CFU/kg	DAI, histological studies, IL-10, IL-17, IgA levels, TNF-α	Alleviated colitis
Ivanovska *et al*[81], 2017	DSS-induced colitis in C57BL/6J mice	*Bifidobacterium infantis* and *Bifodobacterium longum*	Equal parts FOS, GOS and XOS	0.5×10^9 CFU Probiotics and 2.5 g of prebiotics for 1 mo	Gut microbiome, cecal and fecal SCFAs	Increased the diversity of the microbiome and be associated with more SCFAs, and less gut inflammation
Seong *et al* [82], 2020	Chronic restraint stress in male Wistar rats	*L. paracasei*	Opuntia humifusa extract (mucilage + pectin)	*L. paracasei* (1×10^{10} CFU/g) & (10.0/30.0 mg%, w/w) of *Opuntia humifusa* extract once daily for 4 wk	Fecal microbial analysis, serum corticosterone levels, TNF-α levels in the colon tissue	Effective (greater abundance of *L. paracasei* in fecal microbial analysis, lower serum corticosterone levels, lower TNF-α levels in the colon tissue
dos Santos Cruz *et al* [83], 2020	IL 10-knockout mice	VSL#3	Yacon (6% FOS + inulin)	VSL#3 (10^9 CFU/d) + PBY (6% FOS and inulin) for 13 wk	Manifestations of colitis, colon histology, expression of antioxidant enzymes, production of organic acids, and intestinal microbiota	Preservation of intestinal architecture, improve intestinal integrity, increased expression of antioxidant enzymes and concentration of organic acids
Wang *et al* [84], 2019	DSS-induced colitis in C57BL/6J mice	*Lactobacillus acidophilus*, *L. Rhamnosus*, and *Bifidobacterium lactis*	Inulin	Probiotics: 1.0×10^9 CFU per day per mice, and prebiotic 5×10^8 CFU/d	Pathologic scores, mucosal flora	Increased the proportion of helpful bacteria and regulated the balance of intestinal microbiota, reduced the degree of inflammation in acute colitis mice
Son *et al* [85], 2019	DSS-induced colitis in female BALB/c mice	LGG	Tagatose	10^9 CFU/mL of LGG and 25 mg of tagatose once in 2 d for 3 wk	Body weight, food intake, rectal bleeding, stool conditions, blood in stool, expression of proinflammatory cytokines	Effective (gut microbiota composition recovered from the dysbiosis caused by DSS treatment)
Ivanovska *et al*[81], 2017	TNBS-induced colitis in Wistar rats	*L. Casey 01*	Oligofructose-enriched inulin	1 mL containing non-encapsulated probiotic/prebiotics once daily for 2 wk	Assessment of colonic damage, inflammation scoring, MPO and microbiological studies	Effective

DAI: Disease activity index; IL: Interleukin; TNF: Tumor necrosis factor; FOS: Fructooligosaccharides; GOS: Galactooligosaccharides; XOS: Xylooligosaccharides; CFU: Colony forming unit; DSS: Dextran sodium sulfate; MPO: Myeloperoxidase; LGG: *L. rhamnosus strain*; SCFAs: Short-chain fatty acids.

Clinical study of synbiotic among IBD patients

The effects of daily supplementation of total gut repair (TGR) on microbial community composition and activity were investigated in the short-term-Quad-M-SHIME model inoculated with gut microbiota from two individual IBD donors. TGR comprises of probiotics, prebiotics along with combination of amino acids, immunoglobins and flavonoids. TGR supplementation increased SCFA production, increased beneficial bacterial density, decreased inflammation, and damage to the intestinal barrier from endotoxin exposure. Intestinal barrier function was improved compared to controls, and levels of the anti-inflammatory molecules IL-6 and IL-10 were elevated. TGR supplementation daily promoted changes in the gut microbiota of IBD patients.

The efficacy of synbiotic therapy has been evaluated in UC patients in a clinical study (Table 6). Patients received a synbiotic formulation consisting of prebiotic FOS along with 6 probiotic strains. The results showed a significant decrease in inflammation and improved disease status. The double-blind randomized, placebo clinical trial study by Liang *et al*[88] confirmed the efficacy of a synbiotic formulation in suppressing IBD symptoms. The synbiotic consisted of FOS and *L. acidophilus*, *B. bifidum*; *B. longum*; *B. lactis*; and *L. rhamnosus*. Rectal pain, bloating, incomplete bowel movements and diarrhea sensations were significantly improved in patients compared to placebo[88].

A recent randomized, double-blind, controlled trial observed synbiotic use in 18 subjects with functional UC. The treatment included the prebiotics inulin and oligofructose. Sigmoidoscopy inflammation scores were reduced in the synbiotic group compared to the placebo group. The TNF and IL-1α levels in the intestine were also reduced. Furthermore, rectal cultures showed greater epithelial

Table 6 Summary of clinical studies of using synbiotics in inflammatory bowel disease patients

Ref.	Probiotics	Prebiotics	Dose	Parameters analyzed	Conclusion
Amiriani *et al*[86], 2020	*Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptococcus thermophiles*	FOS	Lactocare capsule twice daily for 8 wk	DAI	Mitigated symptoms in patients with UC and suggested to use pre-probiotics in the standard treatment, particularly in those with more than five years of the disease
Kamarlı Altun *et al* [87], 2019	*Enterococcus faecium*, *Lactobacillus plantarum*, *Streptococcus thermophilus* , *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Bifidobacterium longum*	FOS	3×10^9 CFU probiotic and 225 mg/tablet prebiotic for 8 wk	Hemoglobin, leukocyte, neutrophil-to-lymphocyte ratio, sedimentation, and CRP and clinical and endoscopic activity indices	Improvement in clinical activity

DAI: Disease activity index; FOS: Fructooligosaccharides; UC: Ulcerative colitis; CRP: C-reactive protein; CFU: Colony forming unit.

regeneration and reduced inflammation in synbiotic-treated subjects. A tiny, open-labeled trial of 10 active CD subjects, 21 d of 15 g oligofructose and inulin oral administration also showed a substantial lowering of the disease symptoms[89].

Since it has been shown that obesity-induced gut microbiota aggravation can exacerbate IBD symptoms, BG from the Schizophyllum commune, a probiotic, and a synbiotics containing both BG and probiotic (SYN) may improve symptoms of obesity-related colitis. BG and probiotic protected intestinal TJs, but did not modulated the inflammatory markers (*i.e.*, IL-6 and TNF-α) infiltration. In contrast, SYN displayed more prominent actions in attenuating colonic inflammation. SYN treatment group supported the growth of both indigenous and supplemented bacteria while maintaining bacterial diversity, thereby improving the obesity-associated colitis symptoms[90].

SAFETY ASPECTS OF PRO, PREBIOTIC, AND SYNBIOTIC PRODUCTS

The Centers for Disease Control and Prevention advises that over-the-counter prebiotics and probiotics are generally safe for use by healthy people. The ignoring the importance of dose and strain specificity is a concern today. Probiotics manufactured as dietary supplements rather than pharmaceuticals are not subject to regulatory review because they are not required to support claims about the safety or efficacy of food or dietary supplements. This is one of the main reasons for the insufficient or nonexistent information on the efficacy and safety of most marketed products. Probiotic is characterized by a generally safe profile but should be used with caution in certain population groups such as pregnant women, neonates born prematurely, or with immune deficiency. A World Health Organization working group proposed several criteria that are to be considered in order to define strains with GRAS status: Resistance of probioticstrains to antibiotics; evaluation of metabolic properties (lactate production, bile deconjugation) monitoring of side effects in clinical trials, epidemiological studies on the occurrence of side effects after commercial approval, identification of all substances excreted by strains that are toxic to mammals, and determination of the hemolytic capacity of strains[91].

Risks and side effects

No serious side effects of probiotic interventions in IBD patients have been reported. Inadequate immunological stimulation, genes transfer, systemic infections, and fatal metabolic activities have been detected in certain individuals receiving probiotic supplementation. A mild dry cough has been reported in one UC patient with *B. longum* 536 supplementations. Septicemia, and certain cased of endocarditis, have been associated with certain probiotic strains, like *L. acidophilus, L. rhamnosus*, *Bacillus subtilis, S. boulardii, L. Casey,* and *B. breve.* Administration of *Lactobacillus rhamnosus* in 64-year-old UC patient who was treated with prednisone, caused bacteremia due to bacterial transfer from intestinal lumen to the blood. Probiotic interventions have been reported to induce an inflammatory response in the small intestinal region, leading to D-lactic acidosis. The complications in certain patient populations, especially those with compromised immune systems are accelerated by *S. boulardii* and *Lactobacillus GG* administration. Pregnant women, new-borns, and the elderly are at increased risk of potential probiotic infections due to their weakened immune systems[92].

STUDY LIMITATIONS

Significant heterogeneity between studies jeopardizes the interpretation of the current literature on probiotics and prebiotics in IBD. Choice of prebiotic or probiotic studied, the trial design, their doses,

and the outcome were also reported to vary. Study populations varied, with some studies included active disease patients while others working on the remission maintenance persuaded by conventional therapy, antibiotics, or surgery. Most studies enrolled small numbers of patients, limiting their statistical power, which is especially important given the high placebo response rates observed in IBD clinical trials. Finally, no studies provided information on patient diet, which may have a significant impact on the effectiveness of microbial therapy. The exact mechanisms of action have not yet been elucidated. The understanding of the mechanisms responsible for the beneficial effects of probiotics, prebiotics, and synbiotics is rather superficial.

Inadequate evidence on probiotic dosages essential for specific clinical effects has amplified the necessity for molecular description of probiotics to establish health claims. Evidence for immunological mechanisms of probiotics is still limited. Evaluation of interactions between cocktail of probiotic strains in formulations such as #VSL-3 have not been considered and yet to be investigated. Clinical trials and validation studies planned with larger sample sizes require an understanding of the interactions between the microbiota, host, and prebiotic components. Due to the very limited published literature in the field of manufacturing processes and subsequent formulation, much needs to be done to improve strain viability during formulation and storage[93].

OBSTACLES, CHALLENGES, AND FUTURE PROSPECTS

The main barrier with the pre-, pro-and synbiotics is the "difficulty in demonstrating clinical efficacy". The situation is complicated by the different levels of evidence essential to support health claims from country to country. The Food and Drug Administration now states that active ingredients, including probiotics, taken to cure, alleviate, treat, diagnose, or prevent disease must be classified as pharmaceuticals and go through the same approval process as new drugs. Eventually, high-quality human intervention studies are needed to substantiate health claims on products.

Probiotic supplements vary widely in composition, dosage, as well as in host interactions, and these should be specifically considered before recommending their use. Remarkably, several prebiotic and synbiotic products contain a slight amount of prebiotic ingredients (per serving), that may be too small to produce any health benefits. Lower doses are used in part to avoid unwanted GI discomfort, but possibly also for cost reasons. Developing clinically effective synbiotic combinations is a major challenge and must meet several requirements. It is generally expected that the minimum effective dose of each component must be determined.

Maintaining probiotic bacteria viability is a foremost marketing and technical challenge in probiotic applications. A basic prerequisite for probiotics is that the product comprises an adequate number of microorganisms by the expiration date. Therefore, probiotics should cover precise strains and maintain a specific number of viable cells to provide a health benefit to the host. Many viable cultures die during final product manufacturing, storage, transportation, and passage through the gut. As a result, the majority die before consumers can reap the health benefits. Market research has also shown that even before the expiry date, product show much lower count. Therefore, the shelf life of probiotics cannot be accurately predicted. As a result, the industry has to struggle alot to substantiate the label's claims[94].

Also, for optimal effectiveness, probiotics must remain viable after contact with stomach acid, bile, and digestive enzymes to cross the upper GIT. This is a basic property that many products have not tested. Microorganisms may die while passing through the upper intestinal tract to the colon and thus may not be able to colonize the colon. Therefore, they must withstand the gastric acid and bile salts encountered during transit through the GIT.

Even when screening of synbiotic combinations was performed *in vitro* or *in situ*, the methods ignored the environmental factors that influence probiotic strains *in vivo.* Also, competition for the prebiotic substrate between the probiotic strain and members of the gut microbiota was not considered. Identifying prebiotics that specifically and selectively boost the probiotic strain of interest can be challenging.

Clinically effecacious synbiotic development remains a challenge and must meet numerous requirements. It is generally expected that the minimum effective dose of each component must be determined. Including adequate controls in synbiotic studies is particularly challenging. Prebiotic-only and probiotic-only controls must be included, in addition to standard control, for checking the synergistic or additive actions. Justification on how the probiotics and prebiotics were selected and combined should be included[95,96].

FMT IN IBD

For patients with metabolic syndromes linked with gut dysbiosis, FMT is an evolving microbial therapy. The technique involves the transfer of healthy fecal microbe population to patients with metabolic conditions. FMT's technical approach involves oral capsules, nasogastric or nasojejunal tubes, and enemas that are utilized for restoring a healthy GI microbiome. FMT samples are carefully chosen from

healthy donors who have undergone a standard screening procedure for avoiding the risk of transmission of unknown pathogens from donor to recipient. Donors are usually evaluated for their historical backgrounds such as health profiles, family history of autoimmune reactions, metabolic disorders, transfusion details, or any previous surgery. Other donor data include travel history, food intake, particularly alcohol and drugs, and sexual behavior. After selecting an appropriate donor, their stool and blood samples are tested for the presence of pathogens. Extensive support and education are provided to the patients undergoing FMT prior to the treatment. No fecal substances such be present in the colon. Patients may even consume loperamide prior to infusion to make sure that transplanted feces stay there for at least 4 h. The recipient is not allowed to consume antibiotics 48 h prior to infusion[97].

There are several methods of transferring fecal material to the recipients. Presently, fecal material is administered *via* the upper GIT or lower GIT route, or as oral capsules. For patients who are suffering from an inflamed colon, FMT is performed *via* nasogastric tube, esophagogastroduodenoscopy, nasojejunal tube, or upper GI route *via* nasoduodenal tube. Lower GI route FMT can be accomplished by retention enema or by colonoscopy. While colonoscopy aids in the successful recolonization of all the parts of the colon with favorable microflora, retention enemas are only restricted to the distal colon. Retention enemas are however much cheaper and less invasive than colonoscopy. For most reported treatments, FMT patients receive an average relative dose of 25 g *via* the upper GI route compared to an average of 90 g *via* colonoscopy. In an RCT, colonoscopy using a 152 g stool sample reported a 90% success rate in preventing recurrent infections. Another research group reported that consuming 17 g of frozen and thawed or fresh FMT showed 60% efficacy using the retention enema method. The fecal capsules can also aid in restoring ecosystem integrity and overcoming microbial loss in the GI environment[98].

Recently, a study reported that FMT in IBD patients showed a response rate of 53.8% and a complete response rate of 37%. Furthermore, it has been reported that FMT is a more practical treatment with safe and beneficial results for the treatment of active UC patients. Pooled results exhibited that FMT treatment might improve clinical and endoscopic rates of active UC. FMT also significantly alters the microbiota composition of UC patients as compared with control groups[98]. In UC patients, Tian *et al* [99] assessed the B proportion that exhibited a steady rising trend after FMT. *Prevotella* and *Proteus* were also prominently augmented as compared to healthy control. On other hand, it was found that the populations of *Klebsiella* and *Streptococcus*, which are pathogenic bacteria decreased significantly after FMT treatment. Reducing the abundance of *Prevotella* while increasing the proportion of *Klebsiella* and *Streptococcus* was a key factor in the development of UC. Unfortunately, several studies have stated conflicting results, with FMT therapy failing to ameliorate the disease severity and restore the gut microbiota.

In this regard, several studies have found that the efficacy of FMT for treating IBD is unpredictable. Therefore, it is still unclear whether FMT fits into the therapeutic paradigm. Despite reports of significant positive taxonomic changes in the GIT in patients diagnosed with FMT, observations remain conflicting and its functional and metabolic effects are not well documented. For UC, FMT may be a promising treatment, but for CD or pouchitis, very limited information have been available to draw good conclusions[100].

CONCLUSION

Although probiotics and prebiotics have been studied in many animal models and clinical trials of intestinal inflammation and offer health benefits, the individual efficacy of each probiotic strain and its administration remains uncertain. Large, rigorously designed, high-quality human studies need to be evaluated to examine dosage, duration of use, formulations containing one or more strains, and probiotics, prebiotics, antibiotics as well as simultaneous use of substances. For a thorough knowledge of the structure and function of the microbiome with regards to probiotics and prebiotics, modern approaches based on bioengineering, genetic engineering, system biology, molecular biology, multiomics, nanotechnology, and immunology must be employed. These investigations will aid in the comprehension of the relationship between human physiological processes and the microbiome. A potential area for future developments includes implementation of a personalized therapy for IBD, based on a detailed assessment of the gut microbiota and immune system profile in the individuals. Such personalized holistic therapy, that combines biotics with dietary and pharmaceutical therapy, would improve therapeutic efficacy while decreasing adverse effects. This approach will also enable a more thorough understanding of the pathophysiology of IBD and the adoption of targeted therapies for the preservation of the gut microbiome and rectification of bacterial metabolic activities, as well as the restoration of the regulatory immune system. This will enable the use of innovative treatment approaches to manage IBD patients in a safer and more effective manner.

FOOTNOTES

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REVIEW

Changing trends in the minimally invasive surgery for chronic pancreatitis

Raja Kalayarasan, Ankit Shukla

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Abstract

Chronic pancreatitis is a debilitating pancreatic inflammatory disease characterized by intractable pain resulting in poor quality of life. Conventional management of pancreatic pain consists of a step-up approach with medications and lifestyle modifications followed by endoscopic intervention. Traditionally surgery is reserved for patients who do not improve with other interventions. However, recent studies suggest that early surgical intervention is more beneficial as it can mitigate the progression of the pathological process and prevent loss of pancreatic function. Despite the widespread adoption of minimally invasive approaches in various gastrointestinal surgical disorders, minimally invasive surgery for chronic pancreatitis is slow to evolve. Technical difficulty due to severe inflammatory changes has been the major impediment to the widespread usage of minimally invasive surgery in chronic pancreatitis. With this background, the present review aimed to critically analyze the available evidence on the minimally invasive treatment of chronic pancreatitis. A Pub Med search of all relevant articles was performed using the appropriate keywords, parentheses, and Boolean operators. Most initial laparoscopic series have reported the feasibility of lateral pancreaticojejunostomy, considered an adequate procedure only in a small proportion of patients. The pancreatic head is the pacemaker of pain, so adequate decompression is critical for long-term pain relief. Recent studies have documented the feasibility of minimally invasive duodenum-preserving pancreatic head resection. With improvements in laparoscopic instrumentation and technological advances, minimally invasive surgery for chronic pancreatitis is gaining momentum. However, more high-quality evidence is required to document the superiority of minimally invasive surgery for chronic pancreatitis.

Key Words: Robotics; Laparoscopy; Surgery; Chronic pancreatitis; Pancreas; Pancreatitis

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Core Tip: Pain in chronic pancreatitis is a significant symptom that demands utmost attention as it compromises the quality of life and inherently risks narcotic addiction. Surgical management for chronic pancreatitis began with various open surgical drainage and resection procedures. Since pain is the primary indication for intervention, a minimally invasive approach is an attractive proposition in chronic pancreatitis. Despite the slow adoption of laparoscopic and robotic surgery in chronic pancreatitis, safety, and feasibility have been documented in recent studies. The challenges and limitations highlighted in the present review could guide future research on minimally invasive surgery in chronic pancreatitis.

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INTRODUCTION

Chronic pancreatitis is a progressive pancreatic inflammatory disease that leads to fibrosis and parenchymal tissue loss resulting in impaired endocrine and exocrine function. While consumption of alcohol is the leading cause of chronic pancreatitis worldwide, idiopathic pancreatitis remains common in India and China[1,2]. The most common and dominant symptom of chronic pancreatitis is pain, which can be persistent, severe, or recurrent episodes with pain-free intervals significantly impacting the quality of life[2,3].

Traditionally, pain in chronic pancreatitis is managed initially with analgesics, pancreatic enzyme replacement therapy, and lifestyle modifications followed by endoscopic or surgical intervention. Almost half of the patients who do not respond to medical or endoscopic management are referred for surgical intervention[4]. The most common indication for surgery is intractable pain. Studies have reported that early surgical management has better outcomes than intervention in the advanced disease stage[3,5]. While the minimally invasive approach is widely used for various gastrointestinal and pancreatobiliary disorders, its application in chronic pancreatitis is disproportionately low. As chronic pancreatitis is a benign disorder with pain as the primary indication for intervention, a minimally invasive approach is an attractive proposition. The technical difficulty, combined with the potential for vascular injury and bleeding associated with pancreatic inflammation and fibrosis, is the primary reason for the slow adoption of minimally invasive techniques in chronic pancreatitis. However, recent studies have shown the feasibility of laparoscopic and robotic surgery for chronic pancreatitis. Also, with advancements in endoscopic treatment, there is a trend towards the less invasive treatment of chronic pancreatitis. The present review focuses on the challenges, evolution, and changing trends in the minimally invasive management of chronic pancreatitis.

SEARCH STRATEGY

All the authors did a PubMed search of relevant articles. Further, the articles' reference lists were also searched for additional appropriate studies. The keywords and combinations included in the search were: "Pancreatitis"; "chronic pancreatitis"; "idiopathic pancreatitis"; endoscopic management" and "chronic pancreatitis"; "Frey's procedure" and "Laparoscopic"; "Frey's procedure" and "robotic"; "Puestow procedure" and "Laparoscopic"; "Puestow procedure" and " robotic"; "Beger procedure" and "Laparoscopic"; "Beger procedure" and "robotic"; "chronic pancreatitis" and "total pancreatectomy" and " laparoscopic"; "chronic pancreatitis" and "total pancreatectomy", and "robotic". The search was limited to publications in English literature. All the authors agreed that the articles selected for review were relevant.

ENDOSCOPIC MANAGEMENT OF CHRONIC PANCREATITIS

Endoscopic intervention is recommended as a minimally invasive alternative to surgery in patients who do not improve with medical management. The journey of stone removal from the pancreatic duct dates back to 1891 when Alfred Pearce Gould retrieved calculi from the Wirsung duct in a London hospital [6]. Berkeley G Moynihan performed transduodenal removal of pancreatic stones in 1902, followed by transpancreatic stone removal in 1908 by Mayo Robson. The development of the fiberoptic endoscope for diagnosis in 1958 by Basil Isaac Hirschowitz changed the trends of endoscopic management[7]. Watson *et al*[8] succeeded in developing the technique for papillotomy with an energy source leading to

the endoscopic extraction of calculi and subsequent stent placement. Pathogenesis of pain in chronic pancreatitis is multifactorial and includes anatomical and neuropathic factors. Anatomical alterations include ductal hypertension, raised pancreatic parenchymal pressure, acute inflammation, and pancreatic ischemia[9-11]. The lack of correlation between pain severity and anatomical changes suggests neurological factors' role. Neuropathophysiology of pain in chronic pancreatitis includes peripheral sensitization-induced pain, neuropathic remodeling, and central sensitization of pancreatic pain[10,11]. Endoscopic therapy aims to relieve pain by clearance of intraductal stones, thereby decompressing the pancreatic duct. Proponents of endoscopic therapy suggest that patients with complete ductal clearance by endoscopic approach have shown similar pain relief compared to the surgical group[12]. At the same time, the ability to modify the disease progression and prevent loss of pancreatic function with early surgical intervention was proposed in favor of surgical management[13,14].

Traditionally chronic pancreatitis patients were managed initially with analgesics and pancreatic enzyme replacement therapy, followed by endoscopic intervention with or without extracorporeal shockwave therapy[15,16]. Patients with an inadequate clinical response following endoscopic treatment should be discussed in the multidisciplinary meeting and considered for surgical management[10,16]. The potential benefit of this approach is that surgery could be avoided in some patients with successful endoscopic management. However, various studies comparing the efficacy of endoscopic management with surgery have shown better pain control with the early surgical intervention[17-19]. Randomized trials comparing the endoscopic and surgical approaches have shown a better quality of life and pain relief in the surgical group, especially with early surgery[12,20,21] (Table 1). Also, patients with inflammatory pancreatic head mass, distal pancreatic duct stricture, and extensive parenchyma calcifications of the pancreatic head might be difficult to treat by endoscopy. Despite the available evidence favoring surgical treatment, advancements in endoscopic lithotripsy techniques and extracorporeal shockwave lithotripsy (ESWL) are continuously improving the ductal clearance rate, and the endoscopic approach is helpful in a subset of chronic pancreatitis patients[16]. Patients with dominant stricture in the pancreatic head with upstream dilatation and those with intraductal calculi in the pancreatic head or proximal body are ideal candidates for endoscopic intervention.

Pancreatic lithotripsy

Worldwide, ESWL is commonly used for pancreatic lithotripsy, especially in Asia and Europe. While recent advancements like intraductal endoscopic laser or electrohydraulic lithotripsy might improve ductal clearance, they are not widely available. Hence, most of the available data are for ESWL[22]. A meta-analysis of various studies has reported a 70% stone clearance rate with ESWL[23]. With ESWL, stone clearance is more favorable for solitary calculus in the head of the pancreas. However, recurrence of stones after ESWL was seen in 14% to 23% of patients mandating further intervention[24]. ESWL is combined with pancreatic duct stenting in patients with associated pancreatic duct stricture[25]. European Society of Gastrointestinal Endoscopy (ESGE) recommends ESWL for the removal of radiopaque obstructive main pancreatic duct calculi greater than 5 mm found in the head or body of the pancreas and endoscopic retrograde cholangiopancreatography (ERCP) for main pancreatic duct calculi that are radiolucent or smaller than 5 mm[26].

Endoscopic stenting

Main pancreatic duct strictures are often seen in half of the chronic pancreatitis patients and are usually located in the pancreatic head region. The standard management of these strictures is balloon dilatation and placement of a temporary stent for at least a year[27]. Pain relief after a long-term follow-up of 5 years has been seen in almost half of patients after stent withdrawal[28,29]. However, stricture recurrence has been reported in up to 38% of patients after two years[28,30]. Complications related to stenting include stent migration and occlusion. Distal stent migration toward the pancreatic tail and proximal stent migration to the duodenum was reported in 7.5% and 5.2% of patients, respectively[31]. Stent migration can be prevented with large-winged or pigtail catheters[31,32]. The ESGE recommends managing painful pain pancreatic duct strictures with the help of a single 10 Fr stent for one uninterrupted year if symptoms improve after placement[26]. The stent should be exchanged based on symptoms or signs of stent dysfunction on imaging at least six months intervals.

Endoscopic ultrasound-guided intervention

Endoscopic ultrasound (EUS) guided drainage of the main pancreatic duct is used as a second-line procedure after the failure of ERCP. Despite high success rates of 68% to 75%, the complications like perforation, bleeding, and pancreatitis reported in 5% to 43% of patients were key drawbacks for EUS-guided drainage[33,34]. Another EUS-guided procedure is the celiac plexus block, whereby a steroid with a local anesthetic agent is injected at the celiac plexus to block the pancreatic nerve fibers. EUS-guided celiac plexus block is preferred over the traditional percutaneous method. However, despite the high technical success and short-term pain relief in 55%-70% of patients, long-term outcomes are discouraging[35-37].

Table 1 Studies comparing endoscopic and surgical management of chronic pancreatitis

Ref.	Country	No. of patients (*n*), surgery/endoscopy	Complete and partial pain relief (%), surgery/endoscopy	Complication (%), surgery/endoscopy	Mortality (*n*), surgery/endoscopy	Follow up (mo), surgery/endoscopy
Díte *et al* [12], 2003	Czech Republic	36/36	86/61	Not reported	0/0	60
Cahen *et al* [20], 2011	Netherlands	16/15	80/38	0/25	0/1	79
Hong *et al* [19], 2011	China	27/35	77/47	14/22	1/0	60
Kawashima *et al*[17], 2018	Japan	41/10	100/100	20/27	0/0	-
Jiang *et al* [18], 2018	China	40/46	83/80	26/8	0/0	63.5/57.3
Issa *et al* [21], 2020	Netherlands	44/44	58/39	27/25	0/0	18

SURGICAL MANAGEMENT OF CHRONIC PANCREATITIS

Evolution of open surgical procedures

The systemic reviews and meta-analyses of the available studies comparing surgical management with endoscopic intervention continue to support the superiority of surgical treatment in chronic pancreatitis [38-40]. The intriguing voyage of surgical management of chronic pancreatitis has witnessed a huge transformation from surgical decompression of the obstructed duct and denervation of the pancreas to pancreatic head resection, total pancreatectomy, and various other hybrid procedures. In 1911, Link[41], a gynecologist from Indiana, was the first to describe external tube drainage of the pancreatic duct for ductal calculi or stricture with good long-term pain relief. Desjardins and Coffey, around similar times, proposed drainage of the pancreas using the intestine after conducting animal studies[42,43]. This empiricism was materialized by Duval[44] and separately by Zollinger *et al*[45] in 1954 by describing the first caudal end-to-end pancreaticojejunostomy using a Roux-en-Y jejunal loop for chronic pancreatitis. Further, modification in the decompressive procedure was done by Puestow and Gillesby[46], in 1958, by invaginating caudal pancreaticojejunostomy after longitudinally opening the pancreatic duct from the body to the tail region of the pancreas. Their procedure was further modified by Partington and Rochelle[47], in 1960, by creating side-to-side Roux-en-Y pancreaticojejunostomy, commonly called the Puestow procedure. Though the Puestow procedure was considered standard drainage procedure for almost 40 years, the long-term benefits were not befitting. Despite short-term pain relief in 80% of patients, the pain recurred in 30% on long-term follow-up[48-51]. The foremost reason for recurrent pain was undealt nidus of inflammation and persistent ductal disease in the head of the pancreas. To tackle the head disease, German surgeon Hans Beger performed the first duodenum-preserving pancreatic head resection in 1971 and reported postoperative outcomes of 52 patients in 1980[52]. In 1984, Warren described splenopancreatic flap, a denervation procedure for chronic pancreatitis[53]. However, the long-term results of this complex procedure were never published or replicated by other surgeons[9,53].

In 1987, Frey and Smith[54] described a hybrid operation consisting of pancreatic head resection and longitudinal pancreaticojejunostomy, also known as Frey's procedure. Izbicki *et al*[55] modified Frey's procedure by doing a more extensive excavation of the pancreatic head and duct and named it Hamburg modification. Similarly, Gloor *et al*[56] from Berne modified the duodenum-preserving pancreatic head resection by omitting the challenging step of pancreatic neck transection. To minimize the risk of penetrating the posterior pancreatic capsule, Ho and Frey[57], and Frey and Amikura[58] recommended limiting the posterior extent of head coring to the back wall of opened Wirsung and the uncinate duct, also known as modified Frey's procedure. Sakata *et al*[59] described the minimum Frey procedure in which a small spindle-shaped anterior resection of the pancreatic head was performed and reported an equivalent outcome. However, a retrospective study by Tan *et al*[60] reported superior long-term pain relief and quality of life with the original Frey's procedure compared to the modified Frey's. A systematic review and meta-analysis of trials comparing various resectional and hybrid procedures reported similar postoperative pain relief. However, quality of life and other perioperative outcomes favor duodenum-preserving pancreatic head resection procedures[61].

Era of minimally invasive surgery

There is a trend towards minimally invasive procedures for various surgical disorders, and the change is inevitable for chronic pancreatitis[62-64]. Though the surgical procedures described for chronic

pancreatitis are complex and challenging due to inflamed gland they can be accomplished in selected patients[63-65]. In high-volume centers with expertise in advanced laparoscopic procedures, complex pancreatic procedures can be safely performed with comparable postoperative outcomes[63-69]. Also, with its ergonomic advantages, robotic surgery could overcome some of the technical limitations of laparoscopic surgery and potentially widen the use of a minimally invasive approach in chronic pancreatitis[70,71].

Minimally invasive Puestow procedure

Like open surgical procedures, minimally invasive surgery for chronic pancreatitis began with a modified Puestow procedure. Kurian and Gagner[72], in 1999, reported the first series of five patients who underwent a laparoscopic Puestow procedure. Subsequently, two series with 17 and 12 patients were published from India[73,74]. The first small case series of 5 patients from the United Kingdom was published by Khaled *et al*[75] in 2014. The feasibility and favorable short-term outcomes of the laparoscopic Puestow procedure were documented in multiple case series[76-81] (Table 2). In most series, the procedure was performed with five laparoscopic trocars. The initial entry to the lesser sac and exposure of the anterior surface of the pancreas can be technically challenging in patients with recent or recurrent acute episodes of pancreatitis. Hence, those patients should be avoided during the early phase of the minimally invasive Puestow procedure. In most laparoscopic series, two to three gastric retraction sutures are used to lift the stomach away from the pancreas and improve exposure. Needle aspiration is commonly used to identify the pancreatic duct, and intraoperative ultrasound is helpful in patients with undilated duct[74]. Extraction of all intraductal calculi, especially those in the head and tail region, is critical for long-term pain relief. Sahoo *et al*[76] reported the usefulness of cystoscope and endoscopic basket in clearing residual intraductal stones. Proficient intracorporal suturing skill is critical to accomplish safe pancreaticojejunostomy. Kim *et al*[77] reported the benefits of using barbed sutures for laparoscopic pancreaticojejunostomy. Bhandarwar *et al*[78] used endostaplers for laparoscopic pancreaticojejunostomy anastomosis with the anvil part placed within the pancreatic duct. However, as highlighted by the authors, the technique was feasible only in seven out of 17 patients with pancreatic duct diameter of more than 10 mm[78]. Alternatively, a robotic platform can minimize the challenges associated with intracorporeal suturing. After the initial case reports of the robotic Puestow procedure documented its usefulness in a series of seven patients[82-87]. However, with the emerging evidence supporting some form of head resection to achieve long-term pain relief, the minimally invasive Puestow procedure is recommended only in a subset of chronic pancreatitis patients with an atrophic pancreas and dilated pancreatic duct with predominant intraductal calculi.

Minimally invasive Frey's procedure

Frey's procedure is one of the most commonly performed surgeries for chronic pancreatitis. Pancreatic head coring is the technically challenging step of this hybrid procedure, especially during minimally invasive surgery. The first series of laparoscopic Frey's procedure was published by Tan *et al*[88] in 2015. Subsequently, a small series of four patients reported the feasibility and short-term outcomes of laparoscopic Frey's procedure[89]. The largest published series to date had 15 patients in the laparoscopic Frey's group[90]. The relatively small number of studies with fewer patients highlight the technical challenges of laparoscopic Frey's procedure (Table 3). In open Frey's procedure, the surgeon's left hand, kept under the posterior surface of the pancreas head, guides the extent of posterior head coring. In the absence of a definite landmark, pancreatic head coring until the level of the posterior pancreatic capsule is challenging during laparoscopic surgery. Hence, in all the laparoscopic series, only modified Frey's procedure was performed using the main pancreatic duct as the landmark and coring to the posterior wall of the duct[88-90]. In the laparoscopic approach, ultrasonic shears and bipolar vessel sealing devices are commonly used for head coring. Lack of articulation and difficulty securing precise hemostatic sutures further increase the difficulty of laparoscopic pancreatic head coring, especially along uncinate ducts[90]. With its articulating instruments, the robotic platform could potentially overcome technical difficulties during head coring and pancreaticojejunostomy. Hamad *et al*[62] highlighted the usefulness of the robotic approach in bleeding control during head coring in their series of four patients. However, similar to the laparoscopic approach, due to the lack of a definite landmark, coring was limited to the posterior wall of the duct in the robotic modified Frey's procedure (Figure 1). The median operative time and blood loss were 372 min and 163 mL, respectively[62]. The parenchyma posterior to the main pancreatic duct in the pancreatic head was preserved to prevent injury to the superior mesenteric vein[62,91].

Shukla *et al*[92] reported the feasibility of robot assisted Frey's procedure in nine patients with chronic pancreatitis. Robotic approach is associated with less blood loss and shorter hospital stay compared to open Frey's procedure. Tile Pro technology in the robotic platform allows the surgeon to view ultrasound images in the console, thereby avoiding damage to the common bile duct and portal vein during dissection[62]. Bleeding is one of the common causes of conversion in minimally invasive Frey's procedure. Inflammatory pancreatic head mass and preoperative acute exacerbation of pancreatitis were identified as significant risk factors for intraoperative blood loss. Hence, minimally invasive Frey's procedure is recommended in patients with dilated pancreatic duct and enlarged pancreatic head on imaging without inflammatory mass, recent acute exacerbation, and pancreatitis-related complic-

Table 2 Studies on laparoscopic Puestow procedure

Ref.	Country	No. of patients (*n*)	Mean operative time (min)	Mean hospital stay (d)	Conversion (*n*)	Mortality	Follow up, (mo)	Complete pain relief (%)
Kurian *et al*[72], 1999	United States	5	240.0	3-7	Nil	Nil	30.0	80.0
Tantia *et al*[73], 2004	India	17	277.0	5.2	4	Nil	12.0	82.3
Palanivelu *et al* [74], 2006	India	12	172.0	5.0	Nil	Nil	52.8	83.3
Khaled *et al*[75], 2014	United Kingdom	6	278.0	7.0	Not reported	Nil	14.2	66.7
Sahoo *et al*[76], 2014	India	12	265.5	5.8	Nil	Nil	16.5	100 (follow up reported for 8 patients)
Kim and Hong [77], 2016	Korea	11	200.0	7.0	Nil	Nil	21.0	100
Bhandarwar, *et al* [78], 2019	India	28	189.7	5.8	4	Nil	12.0	87.5
Rege *et al*[79], 2019	India	32	131.2	5.2	1	Nil	14.2	75.0
Javed *et al*[80], 2020	India	41	180.0	5.0	Excluded	Nil	43.6	91.0
Nag *et al*[81], 2022	India	33	300.0	7.0	Nil	Nil	25.0	71.0

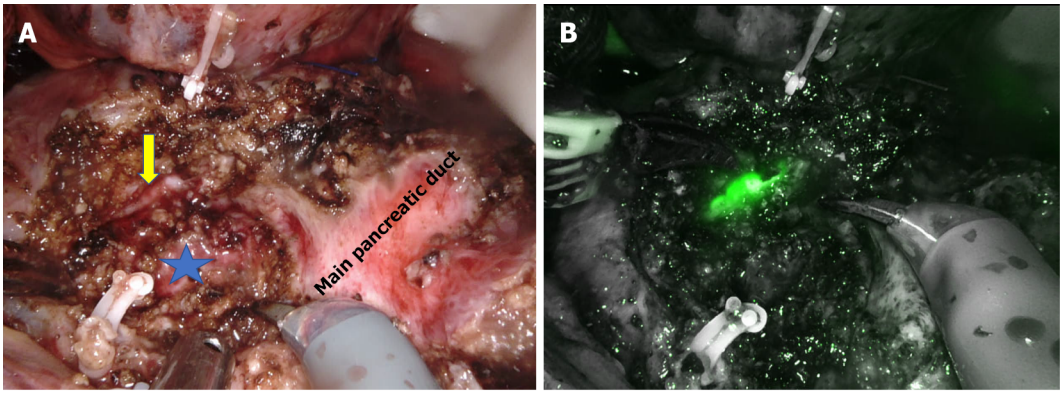
Table 3 Studies on laparoscopic Frey's procedure

Ref.	Country	Patients (*n*)	Mean operative time (min)	Mean hospital stay (d)	Conversion (*n*)	Mortality	Follow up (mo)	Complete and partial pain relief
Tan *et al*[88], 2015	China	9	323	7	2	Nil	3	Not reported
Kilburn *et al*[89], 2017	Australia	4	130	7	Nil	Nil	26	100%
Senthilnathan *et al* [90], 2019	India	15	271	6.4	10 out of 57 patients in different arms	Nil	60	88%

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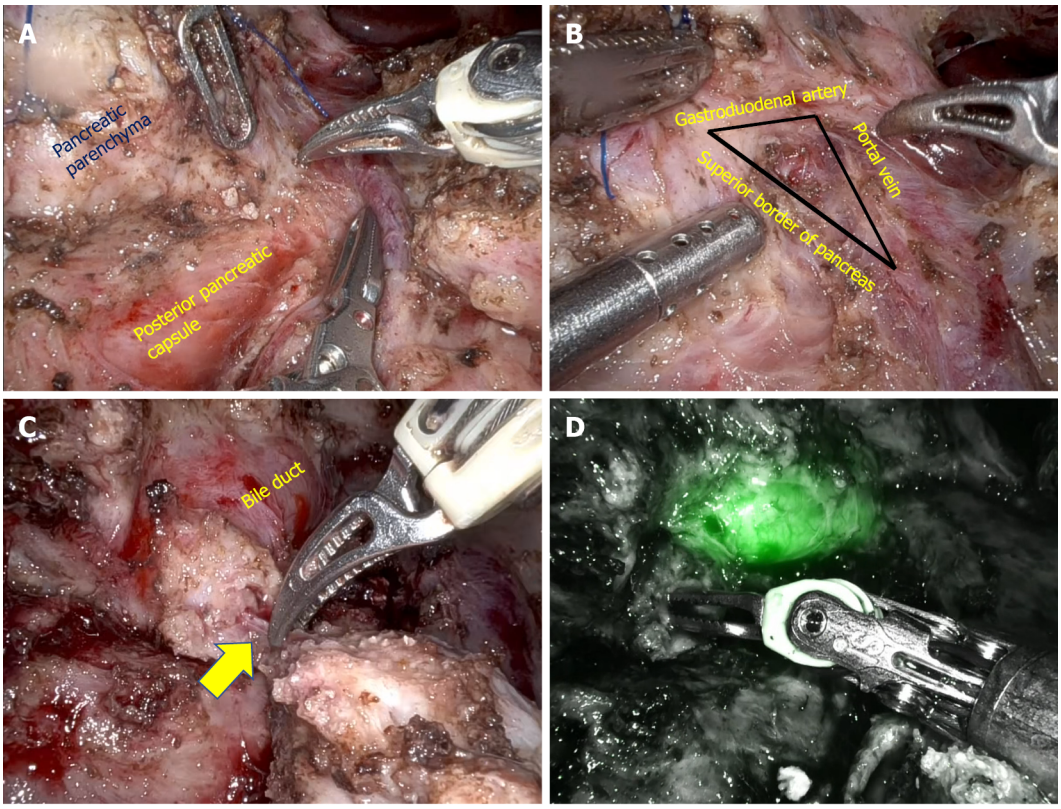
Minimally invasive duodenum preserving pancreatic head resection

In patients with inflammatory head mass, duodenum preserving pancreatic head resection (DPHR) is preferred over pancreatoduodenectomy because of favorable long-term outcomes. As previously highlighted, lack of definite landmarks precludes pancreatic head coring until the posterior pancreatic capsule in minimally invasive Frey's procedure. Minimally invasive duodenum-preserving pancreatic head resection could potentially overcome that limitation. After the initial case reports, it is reported that the feasibility of laparoscopic DPHR procedure in 5 patients with chronic pancreatitis[93-95]. The mean operative time and hospital stay were 275 min and 11 d, respectively. One patient had grade B postoperative pancreatic fistula, and pancreaticojejunostomy anastomotic site bleed in one patient[95]. In laparoscopic DPHR after the pancreatic neck transection, the pancreatic head is retracted to identify the plane between the pancreatic parenchyma and posterior pancreatic capsule. As the dissection proceeds along the superior border of the pancreas, the bile duct should be identified and preserved. Identifying the intrapancreatic duct is a significant technical challenge with minimally invasive DPHR. Indocyanine green (ICG) fluorescence facilitates bile duct identification in the triangle formed by the gastroduodenal artery, portal vein, and superior border of the pancreas (Figure 2). Energy sources should be judiciously used around the bile duct to prevent thermal damage[96-98]. Also, ischemia of the bile duct can be prevented by preserving the posterosuperior pancreaticoduodenal artery, a proximal branch of the gastroduodenal artery, and preserving pancreatic tissue medial to the bile duct. To prevent duodenal ischemia and delayed gastric emptying, the pancreaticoduodenal arcade along the medial border of the duodenum should be preserved. Hong *et al*[95] reported the usefulness of ICG in assessing vascular arcade and identifying common bile duct in a series of 22 patients with different pancreatic pathology. The mean operative time and blood loss of five patients with chronic pancreatitis



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Figure 1 Robotic modified Frey's procedure. A: Pancreatic head coring is done till the level of the posterior wall of the pancreatic duct (marked with star). The bile duct can be seen on the medial wall of the cored-out tissue (arrow); B: Indocyanine green fluorescence demonstrates the bile duct on the medial wall of the cored-out tissue.



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Figure 2 Robotic duodenum preserving pancreatic head resection. A: Dissection of the pancreatic parenchyma from the posterior pancreatic capsule; B: Identification of the common bile duct in the triangle formed by the gastroduodenal artery, superior border of the pancreas, and portal vein; C: Pancreatic duct (arrow) divided at its junction with the bile duct; D: Post pancreatic head resection, indocyanine green fluorescence demonstrates bile duct.

included in their series were 264 min and 215 mL, respectively. The mean postoperative hospital stay was 7.5 d, and there was no conversion to open surgery or postoperative mortality[96].

Inflammation and tissue adhesion in chronic pancreatitis can distort the anatomy of pancreaticoduodenal vessels resulting in vascular injury and significant bleeding during DPHR. A 3D reconstruction of preoperative cross-sectional imaging could help to better understand the anatomy of pancreaticoduodenal vessels and the relationship of the intrapancreatic common bile duct. Also, 3D printing technology can be helpful for surgical training and preoperative planning in patients undergoing minimally invasive DPHR[96-98]. As with other minimally invasive procedures for chronic pancreatitis, a robotic platform could minimize the technical challenges associated with DPHR. Peng *et al*[98] first reported the feasibility of robotic DPHR. However, in a recent series of 68 patients undergoing robotic DPHR for various pancreatic diseases, only three patients had chronic pancreatitis

[99]. Different published series on minimally invasive DPHR highlight technical development and challenges of the procedure in chronic pancreatitis. Minimally invasive DPHR is the preferred procedure in patients with inflammatory head mass and those with enlarged pancreatic head and extensive parenchymal calcifications. However, if the inflammatory changes preclude the safe creation of a retropancreatic tunnel over the portal vein alternative surgical procedure should be considered.

Minimal invasive total pancreatectomy with or without islet cell autotransplantation

Total pancreatectomy is primarily indicated in chronic pancreatitis patients with debilitating pain in whom all other measures are unsuccessful and those with recurrent acute pancreatitis[100]. However, total pancreatectomy should be combined with islet cell autotransplantation to minimize the risk of brittle diabetes. Some centers recommend total pancreatectomy early in the disease course before activation of neuropathic pain circuits, especially in patients with small duct disease or genetic etiology [101]. However, selecting suitable patients is critical as, despite islet cell autotransplantation, more than 50% of patients might require lifelong exogenous insulin. Literature on minimally invasive total pancreatectomy is sparse, with variations in technique[102-105]. Blair *et al*[103], in 2016, reported the feasibility and safety of laparoscopic total pancreatectomy with islet cell autotransplantation in 20 patients with chronic pancreatitis. The mean operative time and hospital stay were 430 min and 11 d, respectively, with no postoperative mortality. Similarly, Fan *et al*[101] reported the feasibility of laparoscopic total pancreatectomy with islet cell autotransplantation in 22 patients with two conversions. In both the laparoscopic series, the pancreatic neck was transected, and two-stage retrieval was used, with the pancreatic head and body retrieved separately[102,104]. However, studies have shown the importance of preserving pancreatic arterial and venous flow until retrieval to reduce warm ischemia time during the pancreatic dissection phase and improve islet yield[105]. In the robotic series reported by Galvani *et al*[102] and Zureikat *et al*[104] the feasibility of total pancreatectomy without pancreatic neck transection and preserving vascular flow till the final step to reduce warm ischemia was documented. Another technical challenge is dense retroperitoneal adhesions due to recurrent pancreatic inflammation. Although laparoscopic and robotic total pancreatectomy with islet autotransplantation is safe and feasible, appropriate patient selection is critical for deriving the benefit of a minimally invasive approach.

CHALLENGES AND FUTURE PERSPECTIVES

The available evidence suggests that minimally invasive surgery for chronic pancreatitis is feasible in selected patients. However, the poor quality of available evidence precludes definite conclusions. Also, the surgeon should adhere to surgery principles for chronic pancreatitis, irrespective of the approach. As the pancreatic head is the pacemaker of pain in most patients, adequate resection and decompression of the pancreatic head are critical. However, most of the reported series on minimally invasive approaches for chronic pancreatitis have focused on the feasibility of lateral pancreaticojejunostomy or modified Frey's procedure which may be appropriate only in a minority of chronic pancreatitis patients. Recent series have shown the feasibility of minimally invasive duodenal preserving pancreas head resection, which may be the ideal procedure for most chronic pancreatitis patients. However, a minimally invasive approach is feasible only in patients without extensive inflammatory adhesions or recent acute exacerbation. Preoperative cross-sectional imaging and biochemical parameters like serum amylase and lipase are not sensitive to predict inflammatory changes. Also, studies evaluating the predictive value of markers of systemic inflammation like white blood cell count, IL-6, and C reactive protein yielded disappointing results[88]. Future studies should focus on identifying reliable markers that can accurately predict ongoing pancreatic inflammation, thereby aiding patient selection for a minimally invasive approach. With recent evidence supporting early surgical intervention before the development of extensive fibrosis or local complications, more patients may be suitable for minimally invasive surgery. Also, the main problem with the existing procedures is they are primarily focused on pancreatic ductal and parenchymal decompression. However, it is well-documented that anatomical factors alone do not contribute to pancreatic pain in all patients. In a subgroup of chronic pancreatitis patients, neurological pathways of pain play a dominant role, which is not addressed by the commonly performed surgical procedures. Also, future studies should compare laparoscopic and robotic procedures for chronic pancreatitis to document the advantages of the robotic platform.

CONCLUSION

As pain is the primary indication for intervention in chronic pancreatitis use of a minimally invasive approach is an attractive proposition. However, due to technical challenges, both endoscopic intervention and minimally invasive surgery for chronic pancreatitis have lagged compared to other benign gastrointestinal orders. With improvements in laparoscopic instrumentation and technological

advances like ICG fluorescence, minimally invasive surgery for chronic pancreatitis is gaining momentum. Also, with its distinct advantages, the robotic platform can widen the adoption of minimally invasive surgery in chronic pancreatitis. However, well-designed trials with long-term follow-ups are required to document the superiority of minimally invasive surgery for chronic pancreatitis.

FOOTNOTES

Author contributions: Kalayarasan R and Shukla A did the literature search; Shukla A wrote the first draft of the review; Kalayarasan R conceptualized the work, supervised the writing, gave intellectual inputs, and critically revised the manuscript.

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REVIEW

Challenges in pediatric inherited/metabolic liver disease: Focus on the disease spectrum, diagnosis and management of relatively common disorders

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Abstract

The clinical scenario of pediatric liver disease is becoming more intricate due to changes in the disease spectrum, in which an increasing number of inherited/metabolic liver diseases are reported, while infectious diseases show a decreasing trend. The similar clinical manifestations caused by inherited/metabolic diseases might be under-recognized or misdiagnosed due to nonspecific characteristics. A delayed visit to a doctor due to a lack of symptoms or mild symptoms at an early stage will result in late diagnosis and treatment. Moreover, limited diagnostic approaches, especially liver biopsy, are not easily accepted by pediatric patients, leading to challenges in etiological diagnosis. Liver dysfunction due to inherited/metabolic diseases is often caused by a variety of metabolites, so precision treatment is difficult; symptomatic treatment is a compelling option for inherited disorders.

Key Words: Hepatitis; Genetic; Liver disease; Cholestasis; Challenge; Pediatric

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Core Tip: The spectrum of diseases causing pediatric liver dysfunction has been changing, and an increasing number of inherited/metabolic disorders have been increasingly recognized as major contributors to liver disease in children. Etiological diagnosis remains challenging due to the frequent absence of symptoms or nonspecific signs and limited diagnostic approaches, especially liver biopsy, which is not easily accepted by pediatric patients. Consequently, the treatment of pediatric inherited/metabolic liver disease is challenging. In this manuscript, we review here the challenges in pediatric inherited/metabolic liver disease, including epidemiological changes in the disease spectrum and challenges in etiological diagnosis and treatment.

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INTRODUCTION

Pediatric liver disease is a common entity in children resulting from different causes, including hepatocyte injury and cholestasis. Recently, the spectrum of diseases causing pediatric liver disease has been changing. Infectious diseases are decreasing due to improvement in hygienic conditions, while the proportions of metabolic disorders, inherited diseases and genetic defects are increasing. At an early stage, elevated liver transaminase may be a unique nonspecific manifestation[1]. Early diagnosis mainly depends on biochemistry, pathogen identification and imaging findings for patients without distinctive clinical features[2]. While new diagnostic methods have improved the diagnostic capability for liver disease in children, some novel challenges remain for pediatricians. Diet and/or lifestyle modifications are usually the primary treatment for some inherited/metabolic liver diseases. Where indicated, drug therapy, surgery and gene modification are necessary to provide lifelong functional correction of liver-based metabolic defects. We review here the changes in epidemiology and the challenges in etiological diagnosis and treatment of inherited/metabolic liver disease in children. Due to the broad spectrum of diseases, we focus on some of the most common conditions resulting in metabolic/inherited liver disease and easily misdiagnosed diseases without evident malformations.

EPIDEMIOLOGICAL CHANGES IN CAUSES OF PEDIATRIC LIVER DISEASE

The exact prevalence of pediatric liver disease is not clear. However, there have been some changes in the disease spectrum causing pediatric liver disease in the past decade. With the popularization of hepatitis B vaccines and the development of effective antiviral treatments, the global incidence of hepatitis B has declined. By 2019, the incidence of hepatitis B in children younger than 5 had fallen from 5% in the 1980s to less than 1% in the 2000s[3,4]. With the promotion of blood screening, the incidence of hepatitis C also declined slightly. By 2018, the global incidence of hepatitis C in children under 18 years old was 0.13%[5]. While the understanding of cholestatic liver diseases (CLD) has deepened in the field of inherited/metabolic liver diseases, an increasing number of noninfectious factors have been recognized as major contributors to liver dysfunction or cholestasis in children[6-10]. A single-center study in China showed that nonviral liver diseases accounted for 23.3% of pediatric liver diseases in 2011-2017, increasing from 17.53% in 2001-2010[11]. Nonalcoholic fatty liver disease (NAFLD) has rapidly become one of the most common liver diseases in the pediatric population. Approximately 2.6% to 9.6% of the population was affected by NAFLD in the Americas and Asia in 2006. The global prevalence of NAFLD in the pediatric population is approximately 10% currently[12]. Recent genetic diagnosis techniques and their easy accessibility raise the possibility of detecting some metabolic diseases involving Wilson's disease (WD)[13], glycogen storage disease (GSD)[14] and α1-antitrypsin deficiency[15]. The incidence of WD is usually quoted as 1:30000, but recent genetic studies have shown a higher prevalence ranging from 1:2400 to 1:6500[16]. CLD affects approximately 1/2500 infants, and biliary atresia accounts for 1/3[17]; genetic or metabolic factors account for approximately one-third of cholestasis cases causing subsequent liver dysfunction, including citrin deficiency (CD)[18], progressive familial intrahepatic cholestasis (PFIC)[19] and Alagille syndrome (AGS)[20]. In the early 2000s, the incidence of CD was 1 in between 17000 and 34000 births[18], while its incidence increased to 1 in 7000 births in 2020[21]. The incidence of GSD is approximately 1:10,000 Live births[22]. AGS had an incidence of approximately 1 in 70000 Live births in 2020[23], an increase from approximately 1 in 100000 in the 1990s[24]. The estimated overall incidence of PFIC increased from 1/300000-1/500000 Live births in the

1990s[24] to 1/50000-1/100000 Live births in the 2010s[25].

CHALLENGES IN ETIOLOGICAL DIAGNOSIS

It is difficult to determine an etiological diagnosis for liver diseases that are undetectable by newborn screening, especially at an early stage. A diversity of inherited/metabolic causes can result in pediatric liver disease[26]. Fatty liver disease (nonalcoholic and alcoholic), autoimmune hepatitis, WD, disorder of bile acid synthesis, AGS, hereditary hemochromatosis, and α-1 antitrypsin deficiencies are among the causes of pediatric liver disease[27]. Hepatocytes have similar responses to different impairments, while clinical and laboratory signs are frequently monomorphic. In addition, the absence of symptoms and nonspecific signs at an early stage contributes to the difficulty of diagnosis.

Liver disease is usually divided into two types - hepatocellular and cholestatic disease - which sometimes overlap. Alanine transferase (ALT), alkaline phosphatase (ALP), aspartate transferase (AST), γ-glutamyl transferase (GGT), prothrombin time, bilirubin and albumin are the most common biochemical markers for liver function, but they are susceptible to extrahepatic factors. A disproportionate increase in the level of ALT and AST compared with ALP suggests hepatocyte damage or muscle disorders; conversely, a disproportionate increase in ALP level compared to ALT and AST indicates cholestatic injury. Moreover, an elevated serum conjugated bilirubin level suggests hepatocellular or cholestatic disease. These markers can assist in roughly sorting the types of pediatric liver disease, but they are not helpful for accurate etiological diagnosis.

With advances in diagnostic tools for pediatric liver disease over the last few decades, some novel diagnostic modalities, such as ultrasound, magnetic resonance, and genetic testing, have been used to evaluate liver disease; however, percutaneous liver biopsy for histopathological evaluation remains crucial for assessment of the severity of hepatopathies[28]. We focus on the challenges in etiological diagnosis in the following section (Figure 1).

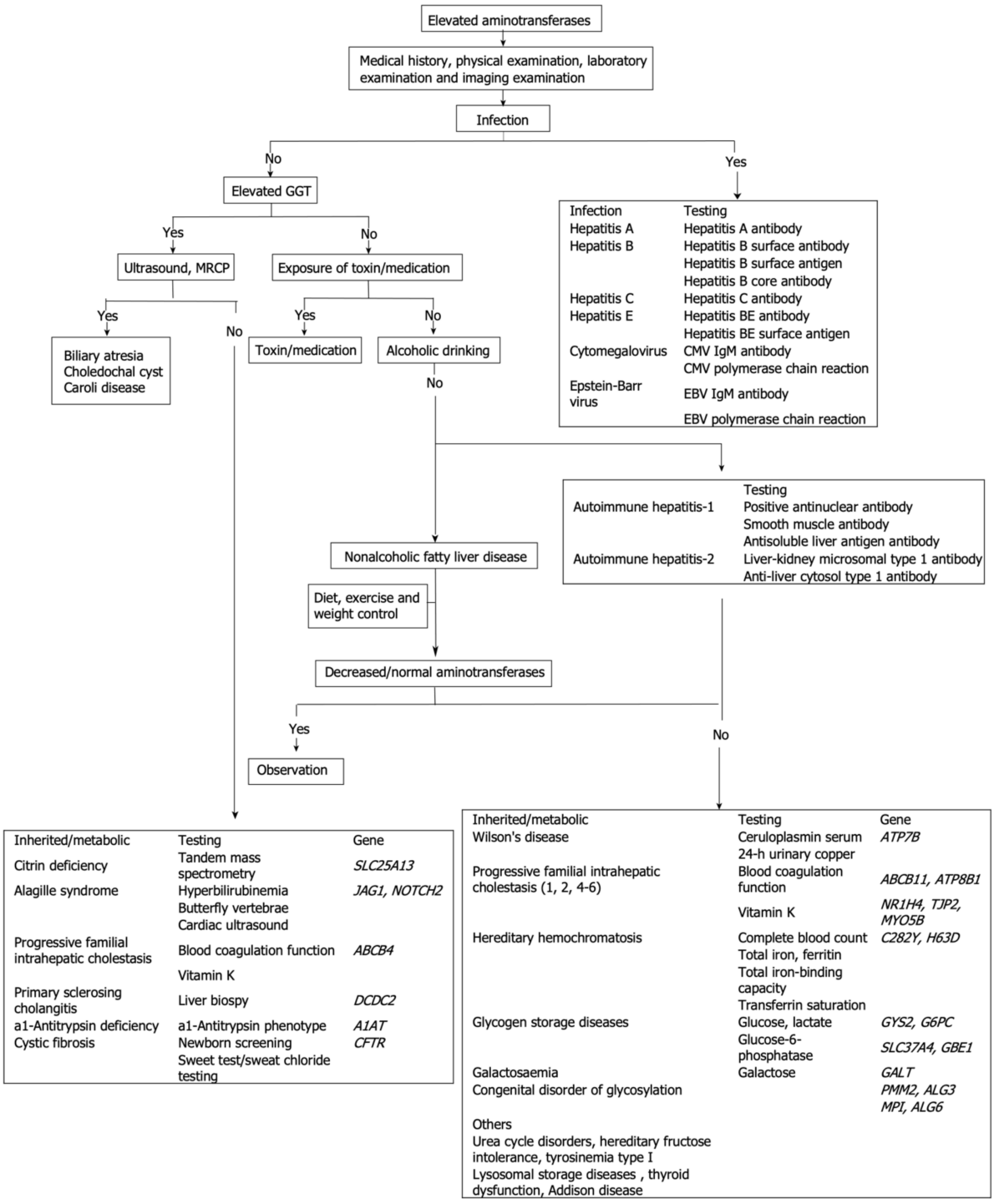
NAFLD

Pediatric NAFLD is frequently free of symptoms, and diagnosis is based on hepatic steatosis on ultrasound or evidence of abnormal liver enzymes and the exclusion of some known causes of hepatosteatosis other than NAFLD. As a standard diagnostic method in NAFLD, liver biopsy has some inherent limitations: the method is invasive; the accuracy depends on the experience of the pathologist; it is difficult to use for NAFLD staging due to the nonuniformity of the small liver specimen; and it may present special challenges in extremely obese children due to difficulty in evaluating the liver location and the depth of subcutaneous fat tissue. Finally, the optimal timing of liver biopsy for diagnosis and follow-up has not been determined[29].

Several noninvasive diagnostic tools have been proposed instead of hepatic histology, including the hepatic steatosis index, NAFLD fatty liver index, liver fat score, and pediatric prediction score, which are widely accepted clinically. However, surrogate markers and scores remain to be validated or are not sufficient to predict the presence of hepatic steatosis[30-32]. Ultrasound technology has been shown to be inaccurate for the diagnosis of hepatosteatosis due to its low specificity and sensitivity[33]. Due to radiation risk, computed tomography is not commonly recommended as a diagnostic tool despite being reasonably specific and sensitive for hepatic steatosis. Despite its accuracy for hepatic steatosis, magnetic resonance spectroscopy and imaging are not widely used due to cost, lack of availability and lack of validated cutoffs to determine NAFLD[34]. As a novel noninvasive tool, transient elastography (TE) has shown promise in the evaluation of steatosis in pediatric patients. TE was reported to perform better than ultrasound for the assessment of hepatic steatosis in children[35]. It is a good diagnostic tool for measuring liver stiffness and can differentiate advanced fibrosis. However, there are some limitations to this diagnostic modality, particularly abdominal adiposity, which increases the distance between the probe and liver; body mass index greater than 30 kg/m^2; and operator experience[36]. Further validation of TE is necessary due to the limited data available for pediatric NAFLD patients. Controlled attenuation parameters are a promising imaging method to detect steatosis based on vibrationally controlled TE. The same probe is used to quantify steatosis by assessing the attenuation of ultrasound in liver fat. Some magnetic resonance-based methods, e.g., magnetic resonance spectroscopy/magnetic resonance proton density fat fraction, have been developed to quantify hepatic steatosis and fibrosis[37]. Its accuracy, cost effectiveness and ability to monitor changes in the level of steatosis in patients with NAFLD remain unknown[38]. Most challenging is that no reliable biomarkers have been identified to detect or predict inflammation in NAFLD. Recently, Chae *et al*[39] successfully developed machine learning-based NAFLD diagnostic models based on metabolome profiles and metabolic pathway changes. It can be utilized as a less-invasive approach for diagnosing the disease.

WD

WD is an autosomal recessive copper metabolic disorder caused by mutations in the ATP7B gene[40]. The clinical manifestations regarding liver involvement range from incidental findings of liver function abnormalities to acute hepatic failure. Symptoms at any age are frequently nonspecific[41]. Early clinical



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Figure 1 Diagnostic flow chart of pediatric liver injury. GGT: Gamma-glutamyl transferase; MRCP: Magnetic resonance cholangiopancreatography.

symptoms in WD patients are diverse and atypical, and biochemical tests have false positives and false negatives, making early diagnosis more difficult. In most cases, its diagnosis is based on a combination of laboratory findings and clinical presentations[40]. The diagnosis might be difficult in children with WD, particularly at early stages of liver disease, given the absence of a single distinctive characteristic or available biochemical test to confirm or exclude the diagnosis. Indeed, many affected children do not have elevated liver copper levels, high urinary copper excretion or a Kayser-Fleischer ring[42].

Serum ceruloplasmin is typically decreased in patients with WD, although normal ceruloplasmin levels are also seen in some patients[40]. Notably, the levels can also be affected by other factors[43]. Therefore, WD cannot be diagnosed solely based on ceruloplasmin levels. Exchangeable copper is an experimental technique that might be able to determine bioavailable copper in plasma, but it does not reliably measure no ceruloplasmin-bound copper levels[44]. Twenty four-hour urinary copper excretion is a useful method to diagnose and monitor treatment in WD; however, it can be lower than 100 μg in

some patients with WD, especially in children and their asymptomatic siblings[45]. Moreover, it might be difficult to interpret because of overlapping findings with other hepatopathies, especially acute hepatic damage or liver failure, and the reference limits vary among laboratories[45]. Although copper quantification in liver tissue has high specificity and diagnostic value, the test is invasive and involves risks; the concentration can be underestimated due to sampling errors or increased in cholestatic disorders[46]. These equivocal findings emphasize the importance of genetic testing for mutation identification. ATP7B gene mutational analysis is an important tool for diagnosing WD. However, it may take time to obtain the results of molecular genetic diagnosis; analysis is difficult due to more than 700 possible mutations, and many patients are compound heterozygotes[47]. Another difficulty of genetic testing research is that, except for a few hotspot mutations, most mutations are extremely low in frequency in the population, making it difficult to collect sufficient cases for statistical analysis, accurate analysis of ATP7B genotype-phenotype and clarification of the corresponding pathogenicity to aid diagnosis. Recently, a scoring system based on all available tests developed by the 8th International Meeting on WD and Menke Disease was found to have good diagnostic accuracy in pediatrics[48].

GSD

GSD refers to a group of inherited disorders of glycogen synthesis, degradation, or metabolism regulation. The hepatic forms usually have a wide spectrum (I, III, IV, VI, IX, and 0) but similar cardinal manifestations[49], although they may have variable expressivity. GSD type I is the most common; type Ia is present in 80% of cases, whereas type Ib accounts for the remaining 20%. The enzyme defect in GSD type Ia is primarily expressed in the liver, kidney, and intestine, while Ib is expressed ubiquitously. Consequently, both types Ia and Ib manifest as hepatomegaly, hypoglycemia, and lactic acidosis, but neutropenia and myeloid dysfunctions are unique to GSD type Ib[50]. GSD classification is sometimes difficult due to the common characteristics among different types. Newborn screening is currently not available for GSD. Encouragingly, some specific screening systems using dried blood spots were reported to be applicable for newborn screening for GSDIa in the real world[51]. A detailed clinical history and careful examination along with laboratory findings could suggest the diagnosis. Reduced enzymatic activity in peripheral blood can confirm the diagnosis of GSD types III, IV and IX; however, normal activity in leukocytes does not exclude the other forms[52]. According to related studies, urinary glucose tetrasaccharide (Glc4) excretion, a biomarker for GSD II, is also elevated in patients with hepatic GSD, so Glc4 might be a good biomarker for GSD, but more studies are still needed to confirm this hypothesis. In pediatric GSD III patients, decreasing Glc4 in urine reflects improved fasting tolerance [53]. Traditional invasive liver biopsy for the assessment of enzymatic activity in hepatic cells is no longer the gold standard and is neither cost effective nor convenient. As a robust alternative diagnostic tool that has emerged over the course of the past few years, gene examination has replaced liver biopsy. It is a noninvasive method for confirming the diagnosis and classification of GSDs, allowing for appropriate specific therapy for different types of GSDs[54]. For many years, the conventional Sanger sequencing method has been the gold standard for the detection and screening of mutations, but this laborious, costly and time-consuming method leads to delayed diagnosis which decelerates care and treatment[55]. Combined with clinical signs and biochemical indices, next-generation sequencing (NGS), as the gold standard, provides a high-throughput and accurate method for genetic diagnoses of GSDs. It bridges the difficulties of GSD diagnosis due to broad genetic heterogeneity and clinical manifestations. The much faster and more sensitive NGS makes clinical application more practicable. However, the identification of variants of uncertain significance poses a challenge in diagnosis owing to variable gene coverage, noncoding and structural variants that are unable to be captured and duplications/deletions that are missed[56]. Higher sequencing coverage increases the validation of findings as well as costs. Therefore, methods with the best accuracy, coverage, and cost are expected to be designed. Finally, traditional static biochemical markers might not adequately reflect the dynamic changes in response to metabolic stressors or treatment. Continuous glucose monitoring appears attractive as a highly informative noninvasive technique to monitor the dynamics of hypoglycemia in patients with GSDs, but it might be limited by regional availability[57].

CLD

CLD covers a spectrum of disorders caused by inborn errors of metabolism, primarily manifesting as a cholestatic syndrome. Intrahepatic cholestasis can be divided into hepatocellular and biliary forms[58]. Hepatic cholestatic disease can cause related clinical symptoms and secondary changes resulting from cholestasis. The diagnosis of CLD is challenging due to the nonspecific symptoms and the broad differential diagnosis. Biochemical indicators include increases in serum ALP and GGT in patients without symptoms at an early stage and an increase in conjugated bilirubin at advanced stages. Isolated elevation of serum GGT and/or ALP can result from certain rare disorders, rapid bone growth or bone disease, and drug intake; consequently, it is limited in the diagnosis of cholestasis due to low specificity. As a sensitive and specific, non-invasive, and relatively inexpensive tool, ultrasonography has some disadvantages in that abnormalities of bile ducts may be misdiagnosed. Moreover, the pancreas and the lower common bile duct are frequently not well depicted. Abdominal computed tomography holds the risk of radiation and might not be superior to ultrasound for biliary tree delineation. Routine new-born screening (NBS) using electrospray ionization tandem mass spectrometry technology has been widely

used for inborn errors of metabolism[59]. Nevertheless, this strategy depends on complete clinical information and therefore is associated with false negatives. In addition, the selection of limited known metabolic pathways likely hampers the discovery of novel metabolic defects[60]. In contrast, NGS panels have recently emerged as an appealing tool to diagnose pediatric metabolic liver disease[60].

CD

CD is the most common inherited autosomal recessive metabolic disorder and is caused by mutations in the SLC25 gene family encoding proteins[61]. There are mainly 3 age-dependent phenotypes[21,61], and neonatal intrahepatic cholestasis (NICCD) is the major pediatric CD phenotype. Neonates or infants with CD manifest with intrahepatic cholestasis, citrullinemia, dyslipidemia, hyperammonemia, galactosemia, and hypoglycemia[62]. In early infancy, these symptoms overlap with those of other CLD, e.g., biliary atresia and neonatal hepatitis, contributing to the difficulty in prompt and accurate diagnosis. Patients with adult-onset symptoms, hyperammonaemia and neuropsychosis can be misdiagnosed with other neurological entities. NBS for CD provides the opportunity to initiate early treatment in newborns with NICCD; however, NBS for CD is not performed in certain countries, and the sensitivity and specificity of NBS results are not satisfactory[63]. False negatives at the cutoff value in NBS have been reported in some studies; less than 30% of NICCD patients were detected by NBS using tandem mass spectrometry to measure amino acids[64]. Abnormal biochemical parameters are important clues for CD diagnosis. The increases in serum transaminase, total galactose, alkaline phosphatase, and direct hyperbilirubin levels, accompanied by the prolongation in coagulation parameters, lead to confusion of the disease with galactosemia and causes misdiagnosis. However, galactose-1-phosphate uridyl transferase activity is normal in these patients[65]. AFP elevation might be an important laboratory finding suggesting NICCD[66]. *SLC25A13* genetic analysis is a reliable method for confirmation of NICCD. However, a percentage of *SLC25A13* mutations are not detectable by conventional DNA analysis alone[67]. The verification of these mutations is challenging for the definite diagnosis of NICCD. Moreover, *SLC25A13* mutations worldwide demonstrate remarkable heterogeneity. In such cases, some reliable evidence for molecular diagnostic strategies originated from sophisticated functional, molecular and in silico analysis of the *SLC25A13* gene and its cDNA, although it is usually labor-intensive and expensive. Sanger sequencing, denaturing high-performance liquid chromatography and PCR restriction fragment length polymorphism are alternative tools for the analysis of gene mutations. However, these methods are too complex to be widely used in clinical practice.

CHALLENGES IN TREATMENT

Challenges in treatment of NAFLD

A universal consensus on drug therapy for NAFLD has not been established. A balanced diet and lifestyle interventions based on exercise are the mainstay of NAFLD management[68]. The side effects of drugs, poor compliance of diet and exercise control, and the risks of surgical therapy remain challenges for pediatricians in the treatment of NAFLD. The provision of low-fat diets and hypocaloric, low-carbohydrate diets, *e.g.*, a diet low in free sugar content[69] or a Mediterranean diet rich in fiber, polyunsaturated fats and antioxidants[70], provide reductions in liver fat content as long as weight loss is achieved. Although proven effective, this treatment modality has some inherent barriers. The patient's compliance, the lack of clinical nutritionists, and the therapeutic recipes juggling the nutrition requirements for development restrict the application. Moreover, no specific diet or program is recommended for the treatment of NAFLD in children from different regions and with different ethnicities. Increasing physical activity is important; however, the appropriate type of physical activity is controversial. There is no general consensus on the exercise category (aerobic, resistance exercise or combined) or the volume and intensity of physical activity. Some studies have demonstrated that combined exercise is more effective than aerobic exercise alone in improving NAFLD progression[71]. Additionally, affected children frequently fail to implement prescribed lifestyle changes due to a lack of motivation to alter contributory habits. The antioxidants metformin and ursodeoxycholic acid, initially used in the treatment of NAFLD in adults, have only limited effects in the treatment of pediatric NAFLD[72]. Similarly, omega-3 fatty acid supplementation was not effective in inducing a significant reduction in ALT or improving liver steatosis on ultrasonography[73]. Some randomized controlled trials indicated that probiotic supplementation might be beneficial in children with NAFLD, but the current evidence does not specify the exact beneficial strain of probiotics, requiring further studies[74]. Interestingly, the strategy of targeting the lysosome has seemed to encourage a new direction for future NAFLD treatment, *e.g.*, inhibition of mTOR localized on the lysosome surface and restoration of normal lysosomal function and autophagy[75]. Nevertheless, all of the studies are limited to animal models.

While bariatric surgery has been indicated as a treatment option in severely obese children and those with comorbidities for the improvement of NAFLD-related liver damage[76-78], the unidentified evidence on nutritional deficiency and its impact on growth and development make it controversial in pediatric patients[76]. Intragastric balloons, a promising alternative temporary physical device, could

improve metabolic parameters and liver alterations in pediatric patients with morbid obesity[79], but further clinical observational evidence is needed for validation in pediatric patients.

Challenges in treatment of CD

Nutritional and medicinal therapy regimens are important for CD patients, and the basic therapy is nutrition. The principle of therapy should be based on the specific food intake[61]. The common therapeutic diet for liver diseases is a high carbohydrate and low protein diet, although it is controversial[80]; other options include a low carbohydrate diet or additional high protein and a low carbohydrate-restricted formula with medium-chain triglyceride supplementation[81]. Dietetic treatment with medium-chain triglyceride (MCT) supplementation and lactose-free or low-carbohydrate formulas has been recommended. The clinical manifestations of NICCD often improve spontaneously within the first year, even without treatment for some patients. Most infants can be reintroduced to protein- and lipid-enriched food by 1 year of age. MCT supplements are suggested even after improvement of clinical manifestations based on the biochemical rationale[64]. In addition to dietary treatment, administration of sodium pyruvate might be effective in correcting growth restriction. However, some subjects develop severe hepatic dysfunction. Liver transplantation (LT) is the most effective option to correct metabolic disturbances in patients with an inefficient therapeutic diet[82]. However, LT also has some disadvantages, *e.g.*, the shortage of liver donors, the cost and the possibility of failing to improve the lives of some CD patients due to immunological complications or other causes. It was reported that supplementation with nicotinamide might be worth trying as a supplemental therapy for CD. mRNA therapy was reported to improve metabolic and behavioral abnormalities in an animal model of CD. Further study is needed to develop safer and more effective treatments for CD patients[21].

Challenges in treatment of WD

The focus of drug therapy for children with WD is to remove excessive copper by promoting copper excretion using chelating agents, blocking intestinal copper absorption with zinc salts[13], or both. At the same time, WD patients should avoid copper-rich foods until they have normal liver biochemistry. D-penicillamine is utilized as a first-line therapeutic medication for acute and/or symptomatic patients with WD. Although it is highly effective, it is linked to serious adverse side effects and requires discontinuation of the drug in some patients[83]. As a second-line drug for patients intolerant to penicillamine, trientine dihydrochloride has equal effectiveness and less frequent adverse reactions, but its high costs limit its application. Trientine tetrahydrochloride is a hybrid with similar benefits and risks to trientine dihydrochloride. A good patient response with a lower dose makes it more cost effective. It has been used for the treatment of WD in adolescents and children older than 5 years old who are intolerant to penicillamine in the European Union, but it was reported that trientine was associated with a higher frequency of initial neurological worsening and increased transaminases[42]. Furthermore, chelation therapies that increase copper excretion can result in iatrogenic copper deficiency[84]. Zinc salts are used for asymptomatic children or in combination with penicillamine for the initial management of symptomatic patients, but some patients display progressive aggravation on zinc therapy[85]. The increased circulating Cu as a result of chelating therapy may lead to oxidative stress and disease deterioration. Melatonin, as an antioxidant, has been shown to strongly ameliorate liver and brain damage from oxidative stress[86]. In fact, treatment failure poses another challenge in the treatment of WD, which can occur with any WD medication early during treatment initiation or later while on maintenance therapy[41]. The main difficulty of WD treatment is to address Wilson's disease crises, including hemolytic crisis and acute liver failure. LT corrects the metabolic defect in the livers of patients with WD and restores normal copper metabolism. The indications for LT are chronic liver disease not responsive to medical treatment, advanced liver failure and fulminant liver failure[13,87]. The fact that acute liver failure is unpredictable, coupled with the shortage in liver donors, remains a challenge to liver transplantation for WD patients.

Challenges in treatment of GSDs

The focus of treatment for GSDs is to maintain blood glucose levels and control lipid and uric acid levels. Diet therapy has remained the primary treatment for GSDs. Cornstarch therapy can better control blood sugar, but infants younger than 6 mo who have not yet fully developed amylase are prone to indigestion and abdominal distention. Starch with a slower rate of absorption may extend sleep periods for children with GSDs. The new waxy maize starch (Glycosade) effectively increases safety through avoidance of overnight feeding and improved sleep and quality of life; however, there have been no dosing guidelines published. Tolerance to the volume required to maintain euglycemia through the night and its strong taste, granular texture, and cost are the main problems. Ketogenic diets were shown to have positive outcomes in the management of patients with some GSD types[88], but the optimal β-hydroxybutyrate concentration in ketogenic diets has not been defined. In addition, high ketones may contribute to osteoporosis, delayed growth, and elevated transaminase[89]. Impaired cortisol levels secondary to deregulation were observed in a cohort study, which could constitute a new potential therapeutic target in GSD I patients[90]. Dietary therapy not aimed at the cause of disease might not

prevent long-term complications and might even trigger secondary metabolic manifestations. Hepatic GSDs are associated with microbial dysbiosis; therefore, supplementation with probiotics is recommended[57]. In recent years, adeno-associated virus (AAV)-mediated gene therapy has moved to preclinical studies and clinical trials. However, current limitations involve the immune response to the AAV product, potential genotoxicity, the need for high vector doses to target nonpermissive tissues, the reduced persistence of vector genomes in diseases with underlying liver degeneration and the need to treat a second tissue other than the liver[91]. LP is the most effective option for GSD treatment, while the shortage of liver donors and the control of postoperative bloodstream infection are major challenges[92].

Challenges in treatment of CLD

Therapy currently focuses on reducing damage to the liver and other organs caused by cholestasis and prevention of the progression of liver fibrosis by reducing toxic substances in hepatocytes. Although medications or dietary modifications are potentially effective for many of these conditions if recognized early, there are some challenges in therapy for CLD. Many metabolic and CLD are sufficiently rare such that pediatricians might have never before seen them.

In some instances, the etiology is unknown, and no specific therapy is available; however, in rare cholestatic disorders in which the cause is known, no specific treatments have been developed[93]. Ursodeoxycholic acid (UDCA) is presently the mainstay of therapy for most cholestatic hepatic diseases; however, all patients with primary sclerosing cholangitis and approximately one in three patients with primary biliary cholangitis do not respond to or intolerant to UDCA treatment[94,95]. As a strong farnesoid X nuclear receptor agonist that downregulates the intestinal bile acid transporter, obeticholic acid is currently the only approved second-line therapy for UDCA nonresponders or intolerance to UDCA; however, only half of these patients respond to obeticholic acid. Patients who do not respond to treatment with UDCA and/or obeticholic acid have an increased risk of progression to biliary cirrhosis, end-stage liver disease and death or liver transplantation[96,97]. Oral vancomycin has been reported to improve liver biochemical tests (ALT and γGTP) and symptoms in children with primary sclerosing cholangitis due to its minimal oral absorption, high concentration in the gut and inhibition of cytokine release from T cells[98]. Further clinical studies of vancomycin treatment for CLD are expected. Although liver transplantation (LT) remains the only curative therapy for patients who fail to respond to conservative management, disease recurrence and extrahepatic manifestations significantly affect quality of life, and long-term studies are needed to understand patient history after LT[99,100]. Hepatocyte transplantation, as an alternative to organ transplantation, is a promising treatment for patients with CLD arising from inherited metabolic disorders, *e.g.*, PFIC[101,102]. The problem of low engraftment rates and long-term survival of transplanted hepatocytes is the most important obstacle regarding current techniques. Moreover, a major limitation is the lack of good-quality donor organs, and thus far, there have been no clinically applicable techniques to monitor the function and survival of engrafted cells[101].

CONCLUSION

Liver function injury in children is etiological heterogeneous. Early etiological diagnosis is critically important because it could help to determine the history of the disease and modify of the treatment regimen, but diagnosis is often delayed due to the presence of nonspecific presentations or the frequent absence of symptoms. It is frequently at the stage of progressive disease when typical symptoms appear. Therefore, recognition leading to diagnosis and treatment remains a great challenge for pediatricians. In this paper, we reviewed the changes in epidemiology and the challenges in the diagnosis and treatment of pediatric inherited/metabolic liver disease. We hope to assist pediatricians in understanding this condition better and identifying and treating this condition as early as possible.

FOOTNOTES

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MINIREVIEWS

COVID-19 related biliary injury: A review of recent literature

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Abstract

Since its emergence in 2019, it has become apparent that coronavirus 2019 (COVID-19) infection can result in multi systemic involvement. In addition to pulmonary symptoms, hepatobiliary involvement has been widely reported. Extent of hepatic involvement ranges from minor elevation in liver function tests (LFTs) to significant hepatocellular or cholestatic injury. In majority of cases, resolution of hepatic injury or improvement in LFTs is noted as patients recover from COVID-19 infection. However, severe biliary tract injury progressing to liver failure has been reported in patients requiring prolonged intensive care unit stay or mechanical ventilation. Due to the timing of its presentation, this form of progressive cholestatic injury has been referred to as COVID-19 cholangiopathy or post-COVID-19 cholangiopathy, and can result in devastating consequences for patients. COVID-19 cholangiopathy is recognized by dramatic elevation in serum alkaline phosphatase and bilirubin and radiologic evidence of bile duct injury. Cholangiopathy in COVID-19 occurs weeks to months after the initial infection and during the recovery phase. Imaging findings and pathology often resemble bile duct injury associated with primary or secondary sclerosing cholangitis. Etiology of COVID-19 cholangiopathy is unclear. Several mechanisms have been proposed, including direct cholangiocyte injury, vascular compromise, and cytokine release syndromes. This review summarizes existing data on COVID-19 cholangiopathy, including reported cases in the literature, proposed patho-physiology, diagnostic testing, and long-term implications.

Key Words: COVID-19 cholangiopathy; Post COVID-19 cholangiopathy; Cholestatic injury; Liver transplant

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Core Tip: Severe cholangiopathy can develop in critically ill coronavirus 2019 patients during recovery, which is reflected by significant derangements in liver function tests and imaging findings consistent with bile duct injury. This condition may progress to acute liver failure, necessitating liver transplantation, and has emerged as a novel indication for transplantation during the pandemic. There are still uncertainties regarding the long-term survival and clinical outcomes of patients who experience incomplete recovery.

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INTRODUCTION

Coronavirus 2019 (COVID-19) infection, caused by severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2), has been a major public health concern in recent years, resulting in significant mortality and morbidity worldwide. Although most patients affected by COVID-19 present with respiratory symptoms and sequelae, extra-pulmonary manifestations, including renal failure, neurological deficits, hepatic injury, and gastrointestinal symptoms, worsening coagulopathy, have been reported. Abnormal liver enzymes are seen in up to 20% of patients and are associated with poor clinical outcomes[1]. In the early stages of infection, aspartate (AST) and alanine aminotransferase levels (ALT) are elevated, followed by cholestatic markers like serum bilirubin, alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) in later stages. Cholestatic liver injury is particularly associated with worse outcomes[2]. This biphasic pattern was described by Hart and colleagues in a study evaluating 496 hospitalized COVID-19 patients[3]. About 6%-20% of patients may have elevation in ALP and GGT at initial presentation. A rise in ALP more than three times the upper limit of normal (ULN) associated with COVID-19 induced cholestatic injury is reported among 1% of critically ill patients[3].

Cholestatic injury in patients ranges from marginal elevation in liver function tests (LFTs) to secondary sclerosing cholangitis (SSC) and liver failure[4]. SSC has been reported in critically ill patients with prolonged hospital stays, patients recovering from sepsis, burns, trauma, and major cardiothoracic surgery.

Post COVID-19 cholangiopathy is a recently described entity considered to be a secondary complication of COVID-19. Currently, there is no consensus on diagnostic criteria for this rare entity. Faruqui *et al*[5] at American Association for the Study of Liver Diseases 2020 described COVID-19 cholangiopathy as severe biliary tract injury resembling SSC seen in patients recovering from severe COVID-19 infection. Diagnosis is made weeks to months after initial admission for COVID-19 infection hence called "post COVID-19 cholangiopathy". These patients often have abnormal LFTs similar to cholestatic injury and bile duct injury on imaging. This review article highlights existing literature on COVID-19 cholangiopathy, including proposed pathophysiology, epidemiology, clinical presentation, treatment, and long-term outcomes[5].

DEFINITION AND DIAGNOSTIC CRITERIA

Although there is no clear consensus on diagnostic criteria for post COVID-19 cholangiopathy, in most studies, patients with severe COVID-19 cholangiopathy were defined as having ALP greater than 1.5 times the ULN, serum bilirubin greater than 2 times the ULN or GGT greater than 3.0 times the ULN[6-9]. These patients often do not have active sepsis or underlying chronic liver disease that may contribute to cholestasis or liver injury. Bile duct abnormalities are noted on imaging.

Magnetic resonance cholangiopancreatography (MRCP) findings include biliary strictures, beaded appearance of intrahepatic bile ducts, biliary dilation, and irregularities of common bile duct, among others (Table 1). A liver biopsy may be needed in some patients to further corroborate the diagnosis. Biopsy findings include cholangiocyte injury, ductal fibrosis, strictures, intravascular microthrombi, *etc.* (Table 1). Endoscopic retrograde cholangiopancreatography (ERCP) may be indicated in some cases to evaluate the bile ducts further and treat biliary strictures or manage choledocholithiasis which may contribute to cholestasis.

Table 1 Liver biopsy and Imaging findings in few previously reported cases of coronavirus disease 2019 cholangiopathy

Ref.	Pathology findings	MRCP
Faruqui *et al*[5], 2021	Acute and chronic large duct obstruction	Beaded appearance of intrahepatic ducts
	Peri-portal fibrosis	Biliary hyper-enhancement
	Cholestasis	
Roth *et al*[6], 2021	Intra-hepatic ductal beading	Beaded appearance of bile ducts along with segments of strictures and dilation
	Biliary strictures and dilation	
	Peri-portal fibrosis	
	Cholangiocyte regeneration	
	Endothelial swelling of hepatic arteries	
Rojas *et al*[7], 2021	Cholestasis	No biliary obstruction
	Peri-portal inflammation	
Daneshjoo *et al*[22], 2020	Enlarged portal tracts	Prominence of intra and extrahepatic bile ducts
	Bile duct epithelial changes	
	Cholestasis, hepatocyte dropout and biliary metaplasia	
	Focal biliary infarcts	
Tafreshi *et al*[17], 2021	Bridging fibrosis	Beaded appearance of intrahepatic bile ducts. Periductal prominence
	Cholestasis and cholangiocyte injury	Normal liver parenchyma
	Bile duct proliferation	
Lee *et al*[16], 2021	Bridging fibrosis	Mild intrahepatic ductal dilation
	Onion skinning of bile ducts and cytoplasmic vacuolization of epithelium	
	Cholestasis	
	Bile duct loss	
	Lymphoplasmacytic infiltration	
Durazo *et al*[8], 2021	Degenerative cholangiocyte injury and cytoplasmic vacuolization	Beaded appearance of intrahepatic ducts with multiple segmental strictures
	Intrahepatic microangiopathy	
	Hepatic artery endothelial swelling, portal vein phlebitis, sinusoidal obstruction	
Cesar Machado *et al*[23], 2022	Cholangiocyte injury	Multi focal strictures and segmental dilation of intra and extra hepatic bile ducts
	Neutrophilic infiltrate	
	Severe cholestasis and fibrosis	

MRCP: Magnetic resonance cholangiopancreatography.

PATHOPHYSIOLOGY OF COVID-19 RELATED BILIARY INJURY

Mechanisms by which COVID-19 results in biliary injury are unclear; however, several hypotheses have been put forth from review of liver biopsies and autopsy studies in COVID-19 patients with significant cholestatic injury (Table 2). One such theory highlights the possibility of bile duct ischemia resulting in cholangiocyte necrosis[10]. Intrahepatic biliary epithelium is often more susceptible to ischemia because of its single source of arterial blood supply from hepatic artery compared to common bile duct and hepatocytes supplied by the portal vein and hepatic artery. Autopsy series by Lagana *et al*[11] and Bütikofer *et al*[12] showed sinusoidal microthrombi with mild hepatic steatosis in patients with COVID-19. Some studies noted the presence of platelet aggregation in sinusoids without gross intravascular thrombi[5]. Contrarily others did not report any evidence of thrombotic injury[5,8,13].

Table 2 Proposed mechanisms of coronavirus disease 2019 cholangiopathy

No.	Proposed pathogenesis of COVID-19 cholangiopathy
1	Cholangiocyte necrosis because of bile duct ischemia[24,25]
2	Micro thrombosis of hepatic sinusoids and ischemic injury[6]
3	Inflammation triggered due to cytokine release (cytokine release syndrome)
4	Direct virus mediated damage[11]
5	Drug induced liver injury in setting of severe COVID-19 infection (antibiotics, Remdesivir among others)
6	Ketamine related cholangiopathy in setting of COVID-19[14]

COVID-19: Coronavirus disease 2019.

Other theories include direct viral damage to biliary epithelium and direct inflammation resulting from cytokine release syndrome. Cytokine release syndrome has been described in patients with critical illness from COVID-19. Release of pro- inflammatory cytokines and mediators results in direct cholangiocyte injury and fibrosis. Angiotensin-converting enzyme 2 (ACE2), the host receptor for SARS-CoV-2, is expressed on respiratory epithelium as well as cholangiocytes. Prior studies evaluating ACE2 expression have concluded that this receptor is more frequently expressed in cholangiocyte clusters (59.7%) compared to hepatocytes (2.6%)[11]. This explains the potential for direct cholangiocyte injury in severe cases of COVID-19. Critically ill patients are often ventilator dependent and receive anesthetics such as ketamine in addition to antibiotics and antiviral therapy. Ketamine associated cholangiopathy has been described in several case reports[14]. Drugs such as remdesivir and amoxicillin-clavulanate are implicated in liver injury, although predominantly hepatocellular damage[15].

CASES OF COVID-19 CHOLANGIOPATHY AND LONG-TERM IMPLICATIONS

To date several authors have reported cases of post COVID-19 cholangiopathy. In the initial published case series, Roth NC and colleagues reported three such cases[6]. These patients developed severe cholestasis after prolonged hospitalization. Biochemical markers consistent with cholestatic injury, persistent jaundice, and liver biopsy findings concerning for moderate peri-portal fibrosis, cholangiocyte injury with microvascular changes were noted in these patients. These abnormalities persisted despite recovery from COVID-19 infection in all patients. No cirrhosis was seen, and none of the patients had prior history of chronic liver disease[6]. Liver transplantation was not required in any of these cases. These patients received hydroxychloroquine, tocilizumab in addition to prophylactic antibiotics during hospital course.

Similarly, Faruqui *et al*[5] reported 12 cases of COVID-19 cholangiopathy. Average time to diagnosis of cholangiopathy was over 100 d in these cases. Bilirubin levels as high as 13 mg/dL and ALP with a median around 1900 U/L was reported. All twelve patients required intubation and three patients required extracorporeal membrane oxygenation (ECMO). Abnormal MRCP findings were noted in all patients. These findings include beaded appearance of intrahepatic ducts and hyperenhancement of common bile duct. Liver biopsy findings in these patients include ductal obstruction as well as mild peri- portal fibrosis without definite duct loss. Majority of these patients were treated with ursodiol without any significant improvement. Liver transplantation was considered in five patients however only one patient received a living donor transplant. One patient in the series developed decompensated cirrhosis with liver and kidney failure at one year[5].

Keta-Cov research group[13] studied 34 patients admitted to intensive care unit (ICU) with COVID-19 pneumonia in a single center in Zurich. Four of the patients in this cohort had persistently elevated cholestatic markers and abnormal imaging. MRCP findings in this group include irregular bile ducts with stricturing. Average time to diagnosis of cholangiopathy ranged from two weeks to over nine months. All patients required mechanical ventilation and were treated with hydroxychloroquine. Ultimately, two of the four patients died. One patient was listed for liver transplant and one patient had stable but persistent disease at one year[13].

Linneweber *et al*[9] reported two patients in Germany who developed biliary injury during recovery from COVID-19 infection. Both patients developed biliary strictures and required ERCP and stent placement. MRCP findings were overall consistent with SSC. One patient remained stable but required multiple ERCPs while the other patient had progressive near complete destruction of intrahepatic bile ducts and died 8 mo after initial diagnosis[9]. Similar findings of SSC were reported in case series by Edwards *et al*[15], Bütikofer *et al*[12], Lee *et al*[16], and Rojas *et al*[7].

Rojas *et al*[7] reported described a case of COVID-19 cholangiopathy in a young female who had a prolonged hospital course for COVID-19. Patient developed jaundice and deranged LFTs 3 mo after her index admission. Peak bilirubin was close to 15 mg/dL and ALP more than 6000 U/L. Interestingly this patient did not have bile duct abnormalities on imaging or ERCP. Patient subsequently underwent a liver biopsy which showed severe obstructive cholestatic picture with periportal inflammation. This patient eventually had improvement in her liver function and did not require a transplant[7].

Durazo *et al*[8] reported a case of COVID 19 cholangiopathy in a 47-year-old obese man who required mechanical ventilation and ECMO in setting of severe COVID-19 infection. Patient developed markedly deranged liver tests two and half months after initial hospitalization. A peak bilirubin close to 20 mg/dL and ALP close to 2700 U/L was noted. Imaging in this case was concerning for diffuse intrahepatic biliary strictures and beading. Pt underwent ERCP. Pt continued to have significant involvement and destruction of intrahepatic ducts and was listed for liver transplant. This patient successfully received liver transplant and had an uncomplicated post op course. Similar cases of successful liver transplant for refractory cholangitis have been reported by Lee *et al*[16], Tafreshi *et al*[17], Blondeel *et al*[18], as well as Rela *et al*[19], among others[17-20]. Allografts in these patients have been reported to be disease free. Cases of persistent liver function abnormalities with stable disease have been reported by a few authors [16,21]. Rate of progression of disease appears to vary among cases with poor outcomes more commonly noted among men, obese patients with metabolic syndromes and more severe COVID-19 illness.

CONCLUSION

Post-COVID 19 cholangiopathy has been described in several case reports and case series. This entity is characterized by severe progressive cholestatic liver injury that can result in liver failure and require transplantation. Progression and onset of disease varies among patients and is not well understood. Individuals with metabolic risk factors and comorbidities who require prolonged ICU stay, mechanical ventilation are at the greatest risk of developing COVID-19 cholangiopathy. It is more commonly reported in men in published literature. At present there is no effective treatment. Hydroxychloroquine, azithromycin and ursodiol have been used in treatment of these patients. Based on follow ups reported in published cases majority of the patients have continued elevation in LFTs, while some progress to liver failure and require liver transplant. In a meta-analysis published by Daneshjoo *et al*[22], the authors concluded that 16% of 30 patients described in the study required liver transplant. Although a rare complication of COVID-19 infection, medical personnel must be aware of this clinical entity. High risk patients should be monitored closely during the recovery period particularly if they suffered a severe clinical course and prolonged recovery. Patients suspected to have COVID-19 cholangiopathy should be referred to liver transplant centers promptly. As time progresses, we will continue to learn more about long term outcomes of those patients diagnosed with cholangiopathy including post-transplant survival and clinical course of those without liver failure however with incomplete recovery.

FOOTNOTES

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ORIGINAL ARTICLE

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Anti-inflammatory effect and antihepatoma mechanism of carrimycin

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Abstract

BACKGROUND

New drugs are urgently needed for the treatment of liver cancer, a feat that could be feasibly accomplished by finding new therapeutic purposes for marketed drugs to save time and costs. As a new class of national anti-infective drugs, carrimycin (CAM) has strong activity against gram-positive bacteria and no cross resistance with similar drugs. Studies have shown that the components of CAM have anticancer effects.

AIM

To obtain a deeper understanding of CAM, its distribution, metabolism and anti-inflammatory effects were assessed in the organs of mice, and its mechanism of action against liver cancer was predicted by a network pharmacology method.

METHODS

In this paper, the content of isovaleryl spiramycin III was used as an index to assess the distribution and metabolism of CAM and its effect on inflammatory factors in various mouse tissues and organs. Reverse molecular docking technology was utilized to determine the target of CAM, identify each target protein based on disease type, and establish a target protein-disease type network to ascertain the effect of CAM in liver cancer. Then, the key action targets of CAM in liver cancer were screened by a network pharmacology method, and the core targets were verified by molecular docking and visual analyses.

RESULTS

The maximum CAM concentration was reached in the liver, kidney, lung and spleen 2.5 h after intragastric administration. In the intestine, the maximum drug concentration was reached 0.5 h after administration. In addition, CAM significantly reduced the interleukin-4 (IL-4) levels in the lung and kidney and especially the liver and spleen; moreover, CAM significantly reduced the IL-1β levels in the spleen, liver, and kidney and particularly the small intestine and lung. CAM is predicted to regulate related pathways by acting on many targets,

such as albumin, estrogen receptor 1, epidermal growth factor receptor and caspase 3, to treat cancer, inflammation and other diseases.

CONCLUSION

We determined that CAM inhibited inflammation. We also predicted the complex multitargeted effects of CAM that involve multiple pathways and the diversity of these effects in the treatment of liver cancer, which provides a basis and direction for further clinical research.

Key Words: Carrimycin; Reverse molecular docking; Network pharmacology; Liver cancer; Anti-inflammatory; Anti-hepatoma

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Core Tip: Although some studies have shown that carrimycin (CAM) has therapeutic effects on inflammation and liver cancer, there are few experimental studies on its component analysis and mechanism of action. The mechanism of action was predicted by reverse molecular docking between the liver cancer target and CAM. By establishing an inflammatory mouse model to assess the state of inflammatory factors after administration, we further proved the therapeutic effect of CAM on inflammation and its possible mechanism in the treatment of liver cancer.

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INTRODUCTION

Carrimycin (CAM), also known as bitespiramycin and shengjimycin, is a new national class I drug that was jointly developed by the Institute of Pharmaceutical Biotechnology of the Chinese Academy of Medical Sciences and Shenyang Tonglian Group Co., Ltd and was approved for listing in June 2019. CAM is a biosynthetic drug produced through genetic engineering technology that was designed according to the characteristics of bacterial drug resistance. CAM catalyzes the formation of isovaleryl side chains by introducing foreign genes, that is, the isovaleryltransferase gene introduced into *Streptomyces spiralis*, which encodes a sixteen-membered ring multicomponent macrolide antibiotic that was obtained by directional acylation of spiramycin (SPM) in microorganisms[1]. Its main components include isovaleryl SPM III, isovaleryl SPM II, isovaleryl SPM I and a certain amount of 9 mixed chemicals, including butyryl-, propionyl-, and acetylspiramycin III and propionyl- and acetylspiramycin II[2]. Structurally, CAM is a macrolide antibiotic. CAM can block the activity of peptidyl transferase in 50S ribosomes to inhibit bacterial protein synthesis to produce antibacterial effects. Moreover, CAM can combine with peroxide scavenging enzymes to induce peroxide generation, destroying biological macromolecules such as DNA and achieving sterilization. In addition, CAM can promote phagocytosis by damaged neutrophils and macrophages in the body. A completed phase III clinical trial showed that CAM is safe for administration, requires a low oral dose, has a low rate of adverse reactions, produces a wide range of antibacterial activities and shows no secondary drug resistance[3]. Thus, CAM has strong medicinal value.

At present, there is a bottleneck in the research and development of new drugs, and using the traditional 'multiple ligands-single target' research and development method makes drug innovation difficult. The cost of discovering a new molecular entity and applying it to the market exceeds $2 billion USD[4]. However, the rate of return is far lower than the investment cost and the time needed. The research strategy of 'multiple targets-single ligand', namely, utilizing reverse molecular docking technology, has effectively solved these problems and become a favorable tool for drug repositioning. Starting from protein receptors and ligands with known chemical structures and using the principles of geometric complementarity and energy complementarity, researchers can calculate the force and affinity of the interaction between small molecule ligands and biological receptor macromolecules and score the results. These scoring results provide the optimal conformation in which a small molecule ligand binds to the biological target macromolecule, leading to prediction of the ideal binding mode between the two molecules. Finally, the corresponding biological targets can be enriched and analyzed to find the most likely disease spectrum. In this study, the distribution and metabolism of CAM in various tissues and organs of mice were assessed by hyphenated to liquid chromatography (HPLC), and the effect of CAM on inflammatory factors in these tissues and organs was detected by enzyme-linked

immunosorbent assay (ELISA). Reverse molecular docking technology was used to screen the targets of CAM, and the predicted target was enriched and analyzed to obtain data on the related diseases. Then, the key targets and associated pathways for the treatment of liver cancer were analyzed by network pharmacology. The molecular mechanism of CAM and the related experimental research in disease treatment are presented in Figure 1. This study provides a reference for relevant research.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade. Chromatographic grade acetonitrile, methanol, and isopropanol, ethyl acetate, ammonium acetate, SPM, roxithromycin, and sodium carbonate were purchased from Aladdin (United States). NaCl, NaOH, and Na_2HPO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. CAM was provided by Shanghai Tonglian Pharmaceutical Co., Ltd.

Assessment of the distribution and metabolism of CAM in various mouse tissues by HPLC

Preparation of solution: A standard solution of CAM was prepared as follows. CAM was diluted to 1200 μL with acetonitrile to prepare a 500 μg/mL solution. This solution was then diluted to 400 μg/mL, 300 μg/mL, 200 μg/mL, 150 μg/mL, 100 μg/mL, 80 μg/mL, 60 μg/mL, 40 μg/mL, 20 μg/mL and 10 μg/mL with acetonitrile. Preparation of the control sample solution was performed as follows. The appropriate amount of roxithromycin was weighed, and acetonitrile was added to prepare a 10 μg/mL mother liquor solution. Then, a total of 980 μL of acetonitrile was added to 20 μL of the solution to prepare a 200 μg/mL sample solution.

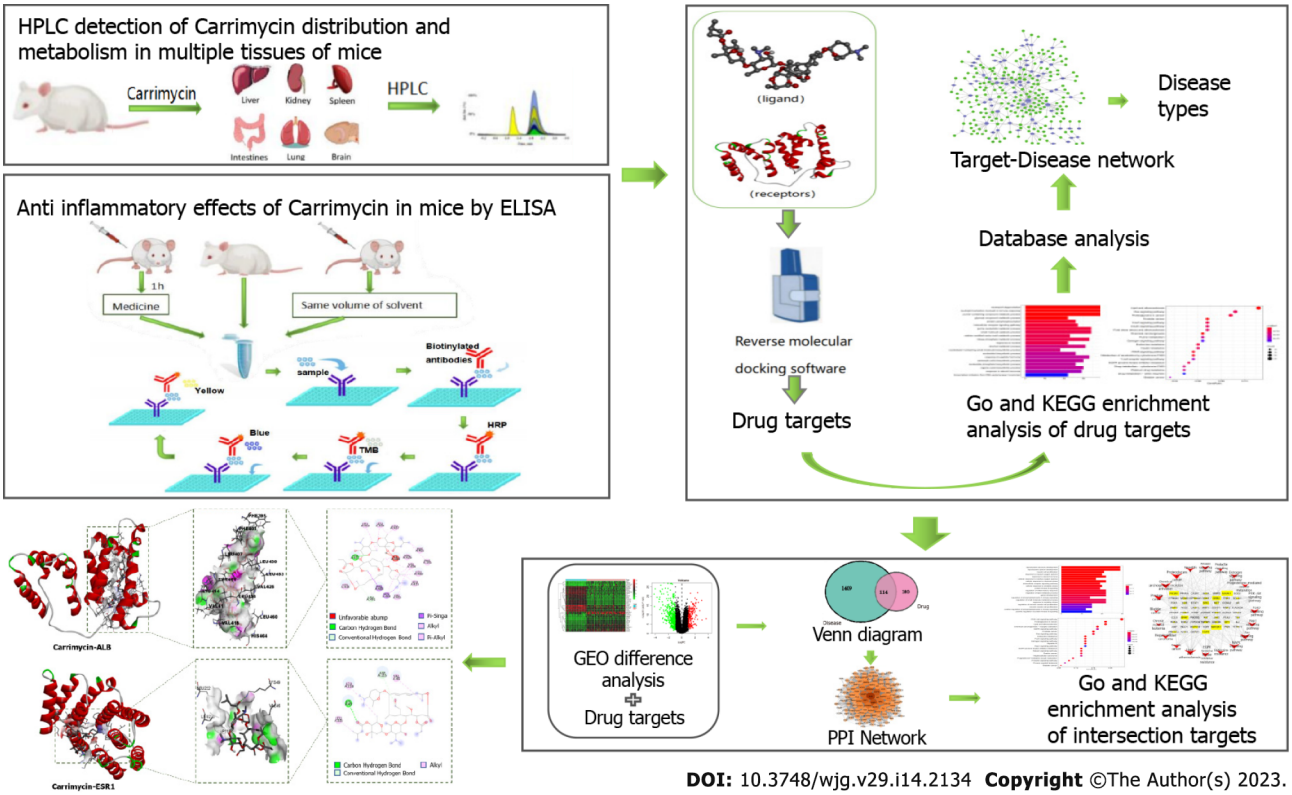
HPLC conditions: HPLC (LC-20AT, Shimadzu, Japan) was used to analyze the chemical composition of the samples using the following parameters: Detector, spd-20a; chromatographic column, Zorbax Eclipse XdB C18 (Agilent, United States); mobile phases, phase A (10 mmol/L ammonium acetate) and phase B (acetonitrile), 50:50 (v/v); flow rate, 1 mL/min; column temperature, 30 °C; and detection wavelength, 232 nm.

Animals and drug administration: Kunming mice were purchased from the Experimental Animal Center of Jiangsu University using animal certificate No. 201927943. A total of 24 mice were used, and they were adapted to the environment in the laboratory before the experiment. The mice were randomly divided into two groups, and a standard curve was constructed. Mice in the administration group were given compound by gavage at one time point, and the second dose was double that of the first dose. The dose administered was 240 mg/kg. Three mice were randomly selected at 0.5, 2.5, 4.5, 12 and 24 h after administration, and their liver, spleen, lung, kidney, intestine and brain were dissected and stored at -80 °C. During the experiment, the mice ate and drank water normally.

Preparation of tissue homogenate: Tissue samples were weighed using LE104E/02 METTLER electronic scales (METTLER TOLEDO Instrument Co., Ltd, China) (lung, spleen, brain, and kidney, 0.3 g), 0.6 mL of acetonitrile was added, and a tissue homogenate instrument (Shanghai Jingxin Industrial Development Co., Ltd, China) was used to prepare the homogenate at a speed of 2800 rpm for 30 s. Pure water (0.3 mL) was added to the homogenate, which was then ultrasonicated (Ningbo Xinzhi Biotechnology Co., Ltd, China) and centrifuged (3500 r/min) for 10 min, and the supernatant was removed for analysis. Tissue samples (liver and intestine, 0.5 g) were used to prepare the homogenate. Pure water (0.5 mL) was added to the homogenate, which was then ultrasonicated and centrifuged (3500 r/min) for 10 min. The supernatant was removed for analysis.

Preparation of the standard curve: First, 100 μL of tissue homogenate was added to 200 μL of water, 50 μL of internal standard (200 μg/mL roxithromycin acetonitrile solution), and 50 μL of standard solution. Two hundred microliters of alkaline reagent (0.05 M sodium carbonate solution) and 2 mL of ethyl acetate-isopropanol (95:5, v/v) extraction solvent were added, and the solution was placed in a vortex shaker (Qilin Bell Instrument Manufacturing Co., Ltd, China). The solution was vortexed for 3 min and shaken at 50 rpm in the dark for 10 min (Changzhou Langyue Instrument Manufacturing Co., Ltd, China), followed by centrifugation (Thermo, United States) for 10 min at 2000 rpm. Two milliliters of the upper organic phase was placed in a 2 mL EP tube (Jiangsu Shitai Experimental Equipment Co., Ltd, China), which was then placed in a metal bath (Scilogex, United States) at 40 °C and dried with nitrogen. The organic solvent was dissolved diluted with 100 μL of mobile phase, vortexed, and injected into the instrument after passing through a 0.45 μm nylon membrane (Jianhu Yadong Glass Instrument Factory, China). The chromatogram was recorded. The standard curve was drawn with the concentration as the abscissa and the peak area/internal standard peak area (AT/AS) as the ordinate.

Sample pretreatment to determine the drug content in tissue: A total of 600 μL of tissue homogenate was placed in a 50 mL centrifuge tube (Corning, United States), 1.2 mL of ultrapure water, 300 μL of internal standard (200 μg/mL roxithromycin acetonitrile solution), and 300 μL of acetonitrile were



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Figure 1 Experimental flow chart. The distribution and metabolism of carrimycin in various mouse tissues and organs were assessed by hyphenated to liquid chromatography, and the effect of carrimycin on inflammatory factors in these tissues and organs was detected by enzyme-linked immunosorbent assay. Reverse molecular docking technology was used to screen for the targets of carrimycin, and the predicted target was enriched and analyzed to obtain data on the related diseases. Then, the key targets and associated pathways for the treatment of liver cancer were analyzed by network pharmacology. HPLC: Hyphenated to liquid chromatography; ELISA: Enzyme-linked immunosorbent assay; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-protein interaction.

added, the solution was mixed, and 1.2 mL of alkaline reagent (0.05 M sodium carbonate solution) was then incorporated. Next, 12 mL of ethyl acetate-isopropyl alcohol (95:5, v/v) was added for extraction, and the solution was vortexed for 3 min and shaken at 50 rpm for 10 min. After centrifugation at 2000 rpm for 10 min, 12 mL of the upper organic phase was taken from 6 centrifuge tubes and combined in a new centrifuge tube. This tube was placed in a metal bath at 40 °C and volatilized with nitrogen for accelerated solvent extraction. When approximately 300-500 μL of liquid remained, the EP tube was vortexed for 10 s, and the liquid was added to a 2 mL EP tube and dried under nitrogen flow. The final sample was dissolved in 100 μL of mobile phase, vortexed, and injected into the instrument after passing through a 0.45 μm nylon membrane, and the chromatogram was recorded.

Data processing: According to the standard curve, we calculated the changes in drug content in various tissues and organs at 0, 0.5, 2.5, 4.5, 12 and 24 h after administration and drew a histogram of these changes in the tissues at different times after administration. Excel 2016 statistical, drawing and calculation software was used.

Anti-inflammatory effect of CAM in mice

Drug preparation: Drug preparation was carried out as follows. Several doses of CAM were added to polyethylene glycol 400 and Tween 80, and the combination was mixed well by oscillation. Then, appropriate amounts of distilled water were added to prepare CAM solutions with high, medium and low concentrations. The model drug was prepared as follows. A 1.82 mg/mL solution of the model drug azithromycin (Aladdin Reagent Co., Ltd) was diluted with a small amount of anhydrous ethanol, and then water was added to produce a solution with a final ethanol content of 10%.

Reagent preparation: PBS (pH 7.35) and Tween 20 were used to prepare a washing solution composed of PBS with 0.05% Tween 20 (if crystals formed in the buffer concentrate, the solution was gently heated until they had completely dissolved). PBS (10 ×) was diluted 1:10 in deionized water to prepare coating buffer (1 ×). The concentrated diluent (5) was diluted 1:5 in deionized water to prepare a 5 × ELISA/ELISPOT diluent.

Standard preparation was carried out as follows. The recombinant mouse interleukin-β (IL-1β) standard was dissolved in distilled water, the volume of which was indicated on the label of the vial. The standard solution was prepared 10-30 min in advance and mixed well to ensure complete and uniform dissolution (concentration of recombinant standard = 1000 pg/mL). Antibody detection was performed by diluting the detection antibody (250 ×) 1:250 in ELISA/ELISPOT diluent (1 ×). The enzyme was prepared by diluting HRP concentrate (100 ×) 1:100 in ELISA/ELISPOT diluent (1 ×).

Establishment of the animal model: Kunming mice were purchased from the Experimental Animal Center of Jiangsu University using animal certificate No. 201929470. A total of 144 mice were used, and they were adapted to the environment in the laboratory before the experiment. Male Kunming mice weighing approximately 24 g were randomly divided into 6 groups, including the normal group, model group, low-dose group (30 mg/kg), middle-dose group (60 mg/kg), high-dose group (120 mg/kg), and azithromycin group (37.9 mg/kg). The effect of each component was assessed at 0 h, 0.5 h, 2.5 h, 4.5 h, 12 h, 24 h, 48 h and 72 h, and three mice were used at each time point. *Staphylococcus aureus* (*S. aureus*) was inoculated into 50 mL of culture medium (washed with PBS and resuspended in 1000 μL of PBS with normal saline. *S. aureus* (3×10^8 CFU·mL^{-1}) was injected into the mice *via* the tail vein at 100 μL *S. aureus*·24 g^{-1} mouse body weight. Drugs were administered one hour later. The mice in the normal group were not administered drugs or injected with bacteria. The mice in the model group were not treated with drugs. The mice in the CAM and azithromycin groups were intragastrically administered 500 μL of the appropriate compound, with a double dose given on the first day. The mice in the model group were given the same volume of solvent. The mice were sacrificed in batches at different time points after administration.

Detection of inflammatory factors: The mice were sacrificed after orbital blood was taken, and each tissue and organ was weighed by an electronic balance. Fifty milligrams of each tissue was placed into a 1.5 mL EP tube, and then 1 mL of precooled PBS and magnetic beads were added. Then, the tissue was homogenized (300 Hz, 30 s). After standing on ice for 30 min, the mixture was centrifuged at 4 °C (10000 g, 10 min), and the supernatant was taken as the test sample. The IL-4 and IL-1β levels in the supernatant were measured according to the instructions of the kit (Thermo Fisher Scientific, China).

Network pharmacology methods

Screening for drug targets and related diseases: First, multiple data platforms, such as the PDB (https://www.rcsb.org) and TTD (http://db.idrblab.net/tt), were used to construct the target protein library of CAM. Docking software, such as INVDOCK and tarfisdock, was used to connect CAM with all targets in the protein library to predict its target. Additionally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. Combining these data with those from GeneCard (https://www.genecards.org), UniProt (https://www.UniProt.org), TCMSP (https://old.tcmsp-e.com/tcmsp.php) and other databases, the predicted target proteins were analyzed one by one, and a relationship diagram was drawn with Cytoscape. This established the target protein-disease type network (degree > 3) to determine the most relevant diseases, which were consistent with the results from enrichment analysis.

Screening for disease targets and drug-disease intersection targets: The most relevant disease from the above analyses was liver cancer, which was selected for further study. Using the GEO database (https://www.ncbi.nlm.nih.gov/geo/) with 'liver cancer' as the search term, the GSE121248 dataset and GPL570 platform were downloaded. logFC ≥ 1 and adj *P* value < 0.05 were the screening conditions to identify the differentially expressed genes. Moreover, the liver cancer-related targets were integrated with the search term 'liver cancer' in the GeneCards database (https://www.genecards.org/), and the genes differentially expressed in liver cancer were combined to obtain a comprehensive list of liver cancer targets. The target gene of CAM in liver cancer was determined by intersection of the target of CAM and the target of liver cancer.

Construction of the protein-protein interaction network and enrichment analysis: The intersecting target gene of CAM and liver cancer was introduced into the STRING database (https://string-db.org) to obtain protein interaction information, and the research species was set as *Homo sapiens*. The lowest interaction score used to obtain the protein-protein interaction (PPI) network was 0.009. Then, Cytoscape 3.7.2 topology analysis of the PPI network was performed, and the core target of CAM in liver cancer was obtained. Then, GO and KEGG enrichment analyses were performed by R software ($P < 0.05$).

Molecular docking verification and visual analysis: ChemOffice software was used to generate the 3D structure of CAM, which was loaded into AutoDock Tools 1.5.6 as the docking ligand. The crystal structures of albumin (ALB) (PDB ID: 1YSX), estrogen receptor 1 (ESR1) (PDB ID: 3HA8), epidermal growth factor receptor (EGFR) (PDB ID: 5Y9T), caspase 3 (CASP3) (PDB ID: 4JJE), and androgen receptor (AR) (PDB ID: 2PIT) were downloaded from the PDB database (http://www.rcsb.org). AutoDock Tools 1.5.6 software was used to remove the water and hydrogen atoms and to perform ligand separation, and the resulting protein structures were stored as the acceptors. AutoDock Vina

1.1.2 was used for molecular docking. The conformation with the lowest binding energy from all docking runs was selected as the docking conformation, and Discovery Studio 2016 Client software was used to visualize the docking conformation.

RESULTS

Distribution and metabolism of CAM in various tissues of mice

There was a good linear relationship between isovaleryl SPM III in the approximate concentration range of 0.1-200.0 μg/mL. The regression equations of the correlation curves in the liver, intestine, kidney, lung, spleen and brain were Y = 0.1977C-0.1072 (R^2 = 0.9953), Y = 0.1929C-0.3317 (R^2 = 0.9970), Y = 0.1447C-0.3909 (R^2 = 0.9982), Y = 0.2512C-0.7149 (R^2 = 0.9942), Y = 0.1748C-0.7313 (R^2 = 0.9966) and Y = 0.2100C-0.9697 (R^2 = 0.9957), respectively, each with an RSD value less than 2%. Thus, the results are reliable and reproducible. The contents of CAM in different tissues and organs 24 h after intragastric administration to mice is shown in Figure 2. The results showed that 2.5 h after CAM treatment, the maximum drug concentration was reached in the liver, kidney, lung and spleen. In the intestine, the maximum drug concentration was reached 0.5 h after administration, and CAM was not detected in the brain.

Anti-inflammatory effect of CAM in mice

IL-4 and IL-1β are common inflammatory cytokines; thus, ELISA was used to analyze the IL-4 and IL-1β levels in different mouse organs and tissues to determine the anti-inflammatory effect of CAM (Figure 3). CAM significantly reduced IL-4 levels in the lung and kidney and especially the liver and spleen. CAM also significantly reduced IL-1β levels in the spleen, liver, and kidney and particularly the small intestine and lung.

Screening of CAM targets and related diseases

From the conformation analysis and scoring results, the targets of CAM were selected. After excluding the ineffective and repeated data, 293 targets were obtained. The 293 predicted target proteins were analyzed by GO and KEGG enrichment analyses. The output GO enrichment analysis results included three pathways: Biological processes (BP) (Figure 4A), cellular components (CC) (Figure 4B), and molecular functions (MF) (Figure 4C). The core targets of CAM are mainly involved in the response to neutrophil degranulation and the immune response and in neutrophil activation, purine-containing compound metabolism, glycosyl compound metabolism and protein autophosphorylation. The molecular functions mainly involve carboxylic acid binding, organic acid binding, protein tyrosine kinase activity and serine endopeptidase activity. In terms of cell composition, the main targets involved vesicle cavity, cytoplasmic vesicle cavity, and collagen-containing extracellular matrix (ECM) pathways. The KEGG enrichment pathway analysis results in Figure 4D demonstrate that the pathways in which CAM participates in disease treatment mainly include the Ras, FoxO, and insulin signaling pathways.

The 293 predicted target proteins were analyzed one by one, and the data from multiple databases were combined to screen 77 main targets (circles) and 22 main diseases (rectangles) (Figure 5). The targets with the highest degrees were EGFR (degree = 7), mesenchymal epithelial cell transforming (MET) (degree = 7), matrix metalloproteinas (MMP)-2 (degree = 7), and ESR1 (degree = 6). The main diseases involved were liver cancer (degree = 18), breast cancer (degree = 13), lung cancer (degree = 12), and prostate cancer (degree = 10).

Screening liver cancer targets and their intersection with those of CAM

First, 580 differentially expressed genes in liver cancer were screened using the GEO database to construct a volcano plot (Figure 6A). Genes with significant upregulation and downregulation were selected to draw a heatmap (Figure 6B). Among them, 167 differentially expressed genes were upregulated in hepatocellular carcinoma, such as AKR1B10, SPINK1, ROBO1, CCL20, and RRM2, and 413 differentially expressed genes were downregulated in hepatocellular carcinoma, such as SRPX, CLEC4 M, CLEC4G, CLEC1B, OIT3, and CRHBP. After integration with the liver cancer-related targets from the GeneCards database, a total of 943 liver cancer-related targets were retrieved, and duplicate values were removed from the combined dataset to obtain 1523 liver cancer disease targets.

R software was used to match and map 294 CAM target genes with the 1523 liver cancer disease targets, resulting in 114 intersecting target genes, including transthyretin, bone morphogenetic protein 2, ALB, Glutathione S-transferase P1, PNP, and PLG. These results are shown in the Venn diagram in Figure 6C.

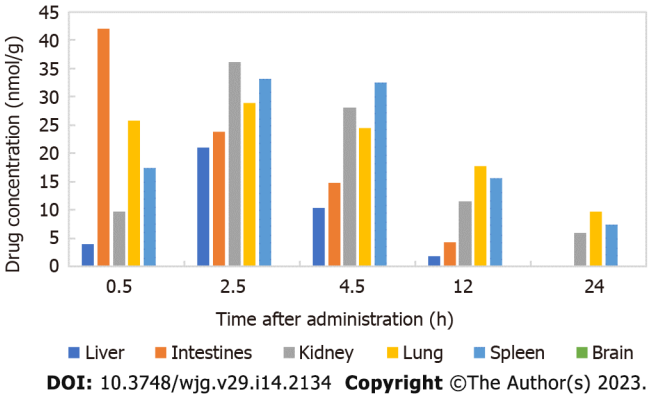
Establishment of the PPI network and enrichment analysis

The obtained PPI network is shown in Figure 6D. The PPI network was used with Network Analyzer in Cytoscape v3.7.2 for degree of freedom, mediation and centrality algorithm topology analysis to select the greater than the average targets as the key targets. Only the top 15 targets were retained (Table 1).

Table 1 Key target genes of carrimycin in the treatment of liver cancer

Gene name	Degree	Betweenness centrality	Closeness
ALB	110	0.04074405	0.97413793
ESR1	102	0.02219136	0.91129032
EGFR	97	0.01545146	0.87596899
IGF1	95	0.01457077	0.86259542
HSP90AA1	93	0.02011442	0.84962406
CASP3	93	0.01307351	0.84962406
AR	91	0.01215915	0.83703704
SRC	90	0.01137002	0.83088235
PPARG	89	0.01582869	0.82481752
MAPK1	88	0.00943128	0.81884058
IL2	86	0.01154328	0.80714286
SOD2	84	0.01534439	0.79577465
MAPK14	84	0.00918383	0.79577465
NOS3	83	0.0095422	0.79020979
MMP9	83	0.00820256	0.79020979

ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; IGF: Insulin-like growth factor; HSP: Heat shock protein; CASP3: Recombinant caspase 3; AR: Androgen receptor; SRC: Sarcoma gene; PPARG: Peroxisome proliferator activated receptor gamma; MAPK1: Mitogen-activated protein kinase 1; IL: Interleukin; SOD: Superoxide dismutase; NOS: Nitric oxide synthase; MMP: Matrix metallo proteinase.



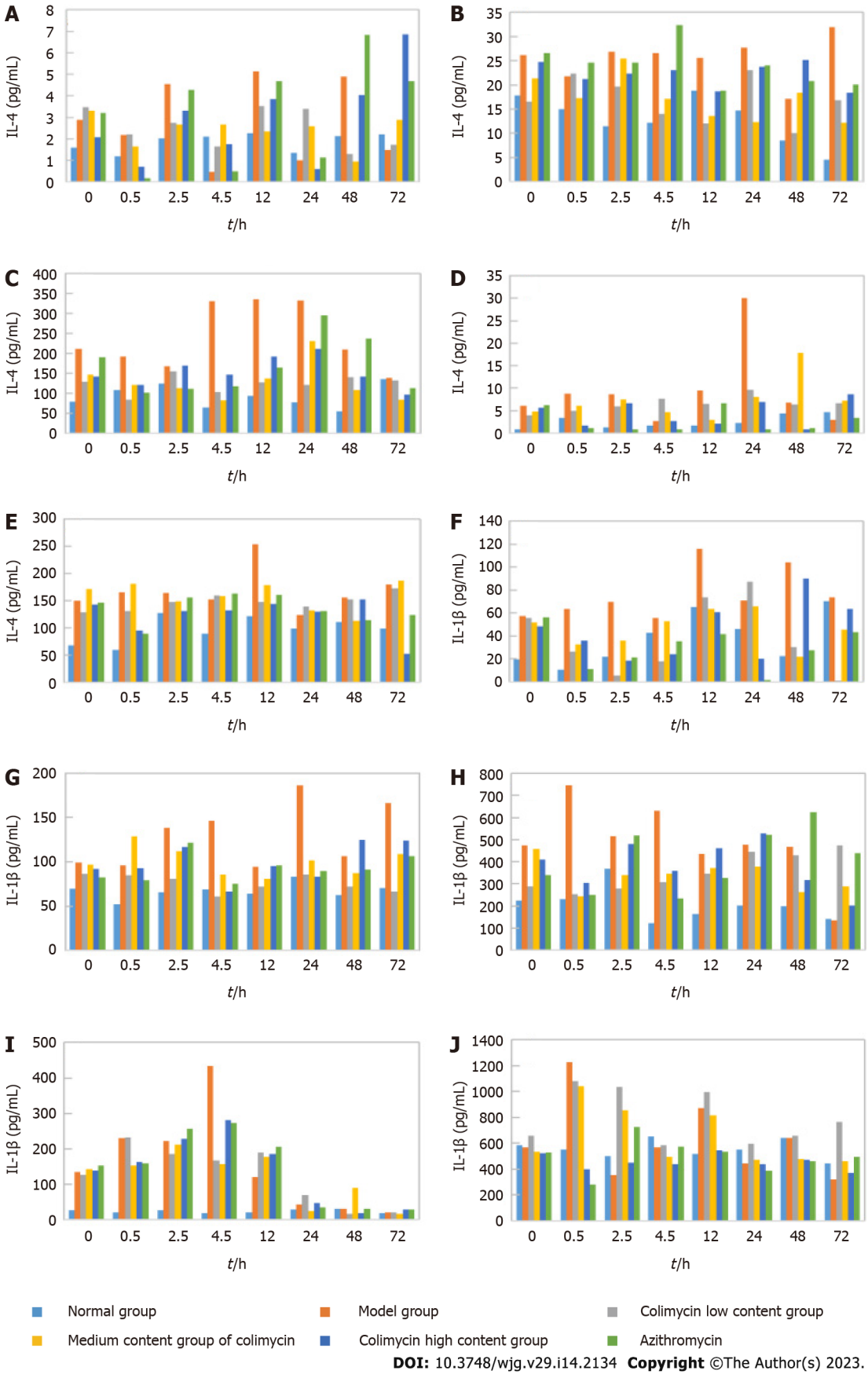
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Figure 2 Concentrations of carrimycin in different tissues and organs after intragastric administration to mice (nmol/g). 2.5 h after carrimycin treatment, the maximum drug concentration was reached in the liver, kidney, lung and spleen. In the intestine, the maximum drug concentration was reached 0.5 h after administration, and carrimycin was not detected in the brain.

Finally, we screened the core target proteins of CAM in liver cancer: ALB, Insulin-like growth factor 1 (IGF1) (somatomedin C), ESR1, EGFR, AR, mitogen-activated protein kinase 1 (MAPK1), MAP2K1 (gene encoding mitogen-activated protein kinase 1), CASP3, *etc.*

The GO enrichment analysis results included the terms BP (Figure 7A), MF (Figure 7B) and CC (Figure 7C). The core targets of the active components of CAM were mainly enriched in processes including reproductive structure development, reproductive system development, muscle cell proliferation, response to reactive oxygen species, cell response to reactive oxygen species, molecular functions, nuclear receptor activity, transcription factor activity, and ligand activation, while the cellular components included vesicle cavity, secretion granules, cytoplasm vesicle cavity, membrane microregion, membrane region and ECM.

KEGG pathway enrichment analysis of the key targets of CAM in liver cancer performed in R resulted in 140 signaling pathways ($P < 0.05$). The top 20 pathways were selected to create a bubble map (Figure 7D). The main pathways involved in CAM treatment of liver cancer include the PI3K-Akt, MAPK, Ras, FoxO, estrogen, and hepatitis B pathways and others. CAM acts on multiple targets in the



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Figure 3 Anti-inflammatory effect of carrimycin in mice detected by enzyme-linked immunosorbent assay. A: Interleukin-4 (IL-4) expression in the intestine; B: IL-4 expression in the lung; C: IL-4 expression in the liver; D: IL-4 expression in the spleen; E: IL-4 expression in the kidney; F: IL-1β expression in the intestine; G: IL-1β expression in the lung; H: IL-1β expression in the liver; I: IL-1β expression in the spleen; J: IL-1β expression in the kidney. IL: Interleukin.

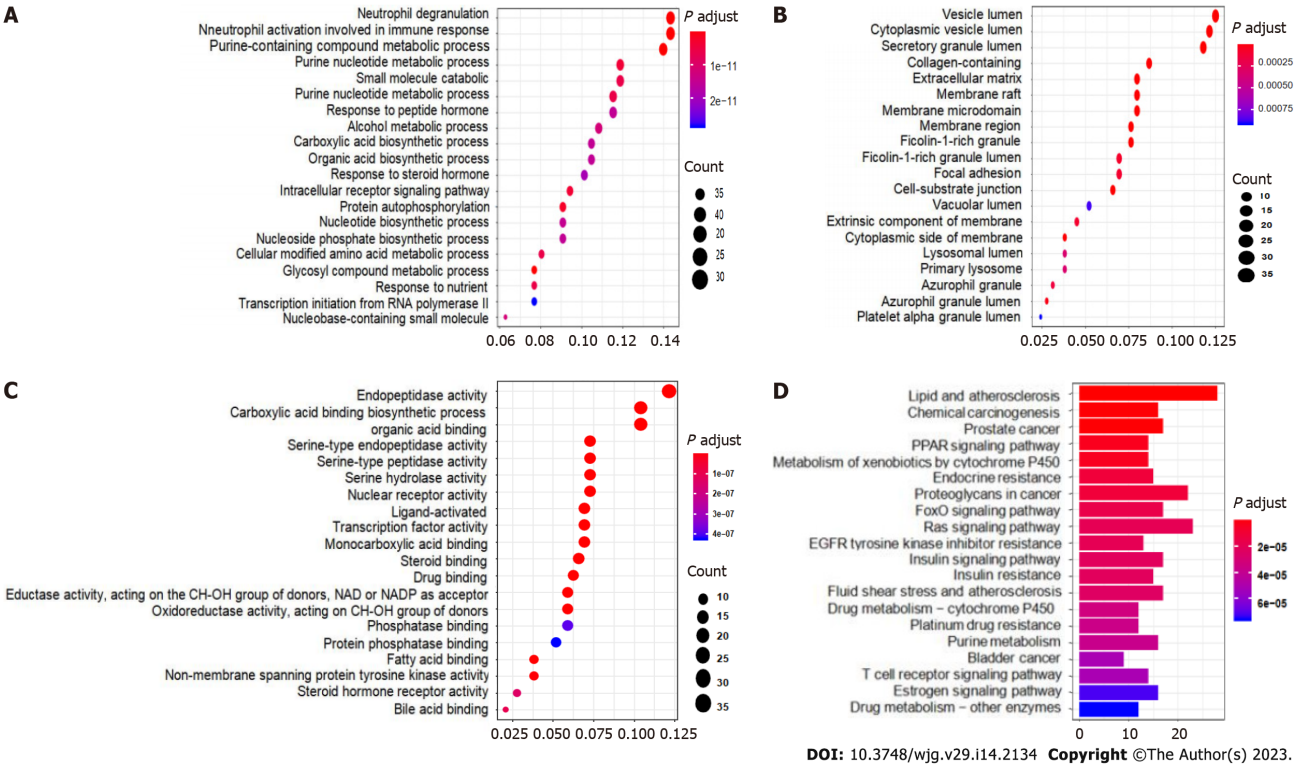


Figure 4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses of the targets of carrimycin. A: Biological processes; B: Cellular components; C: Molecular functions; D: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of carrimycin showing that the main pathways in which carrimycin is involved in the treatment of diseases include the Ras, FoxO, and insulin signaling pathways. PPAR: Peroxisome proliferator-activated receptor.

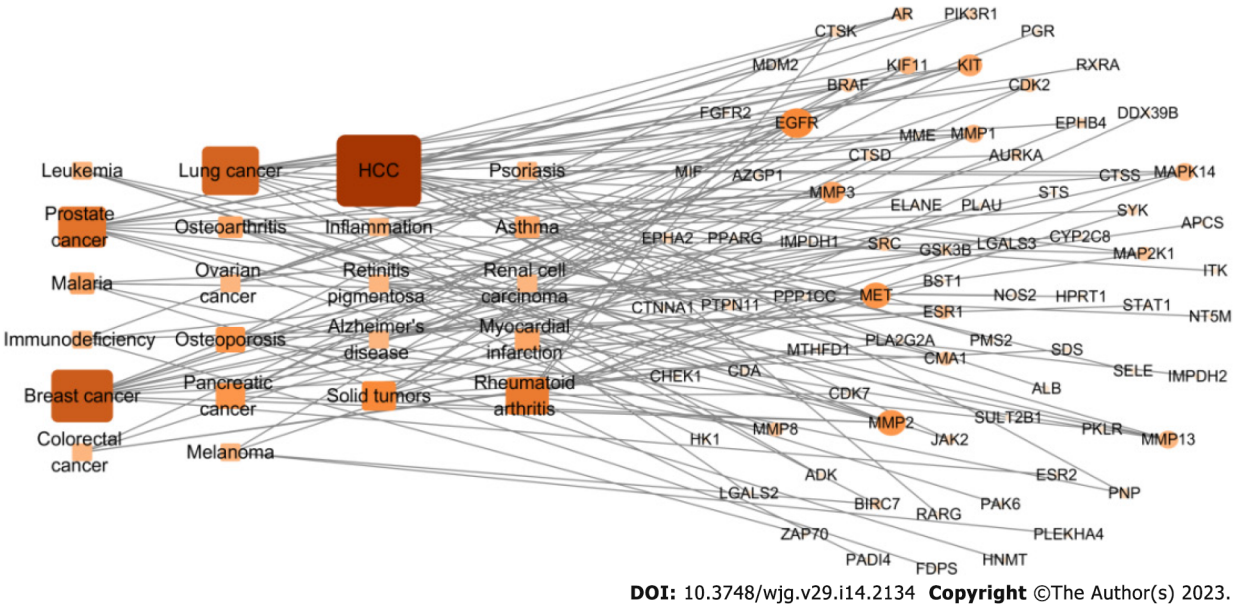
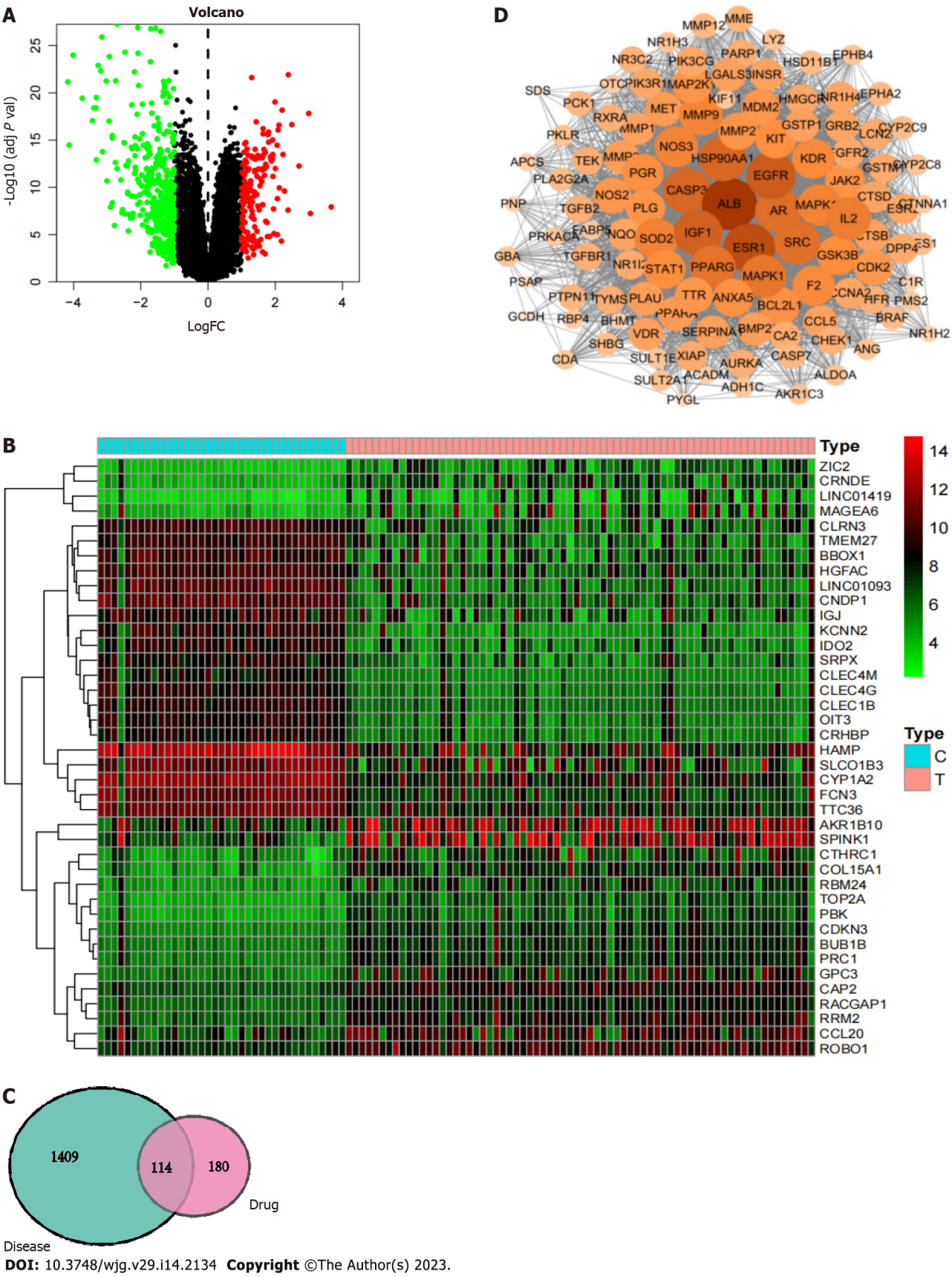


Figure 5 Target-disease network of carrimycin. The 293 predicted target proteins were analyzed one by one by combining multiple databases to screen 77 main targets (circles) and 22 main diseases (rectangles). The main diseases involved were liver cancer (degree = 18), breast cancer (degree = 13), lung cancer (degree = 12), and prostate cancer (degree = 10). HCC: Hepatocellular carcinoma.

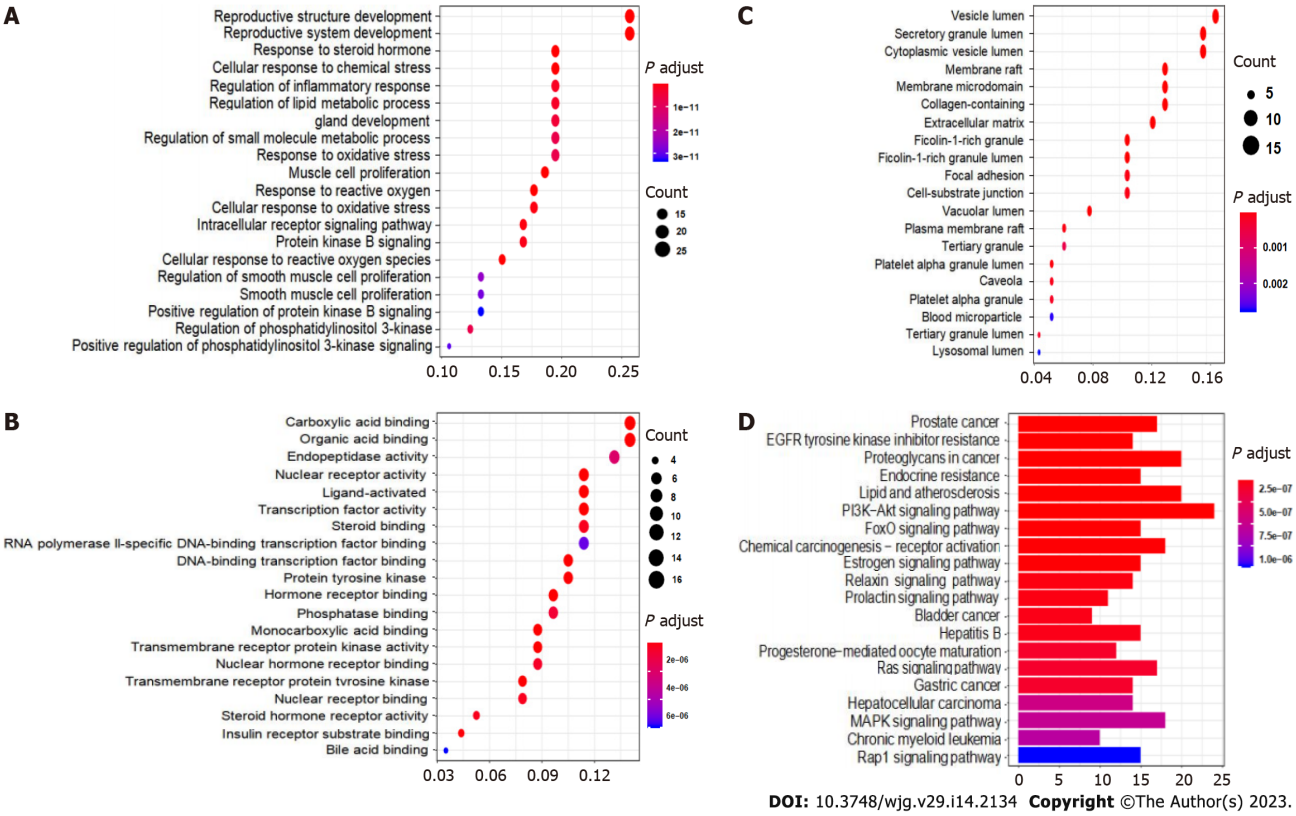
PI3K-Akt signaling pathway to produce effects against liver cancer by activating or inhibiting certain proteins in this pathway (Figure 8A).

According to the relationships between the key targets of action and the signaling pathways, a key target-pathway network map was constructed using R language and Cytoscape v3.7.2 (Figure 9). The graph has 80 nodes and 307 edges. The nodes include 60 related targets indicated by yellow rectangles and 20 signaling pathways indicated in red type V. The node size corresponds to the degree value; the



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Figure 6 Network pharmacology illustration. A: Differential gene volcano plot; B: Differential gene heatmap. Up- and downregulated differentially expressed genes can be derived from volcano plots and heatmaps; C: Venn diagram of the common targets of carrimycin and liver cancer. The common disease-drug targets were determined from this map; D: Protein-protein interaction network of carrimycin and acquisition of the core targets of carrimycin in liver cancer.



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Figure 7 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis results of the intersecting targets. A: Biological processes; B: Molecular functions; C: Cellular components; D: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the key targets of carrimycin in the treatment of liver cancer. The main pathways involved include the PI3K-Akt, MAPK, Ras, and FoxO signaling pathways.

larger the node is, the more important this factor is the network. Therefore, CAM can regulate multiple biological pathways and treat liver cancer by acting on multiple targets, such as MAPK1, MAP2K1, phosphoinositide-3-kinase, regulatory subunit 1, EGFR and MET.

Molecular docking of CAM with key targets

In molecular docking, it is generally thought that a stronger affinity between a ligand and a receptor gives a lower binding energy, and hence, a greater probability of interaction. When the obtained binding energy is less than zero, the receptor and ligand can bind spontaneously. The binding energies for each receptor-CAM interaction are shown in Table 2. All of the binding energies are lower than -5.0 kcal/mol, indicating that CAM has a good ability to bind to these targets. To further study these interactions, the relationships between CAM and these targets were plotted and analyzed (Figure 10).

DISCUSSION

In this study, reverse molecular docking and network pharmacology were used to screen the targets of action and related diseases of CAM, analyze the function and pathway enrichment of the targets of action, and establish a network of the key target proteins in disease to determine the most relevant disease types from the enrichment analysis results. In addition, the distribution and metabolism of CAM and its effect on inflammatory factors in various tissues and organs were examined, and the mechanism by which CAM can treat disease was systematically explored.

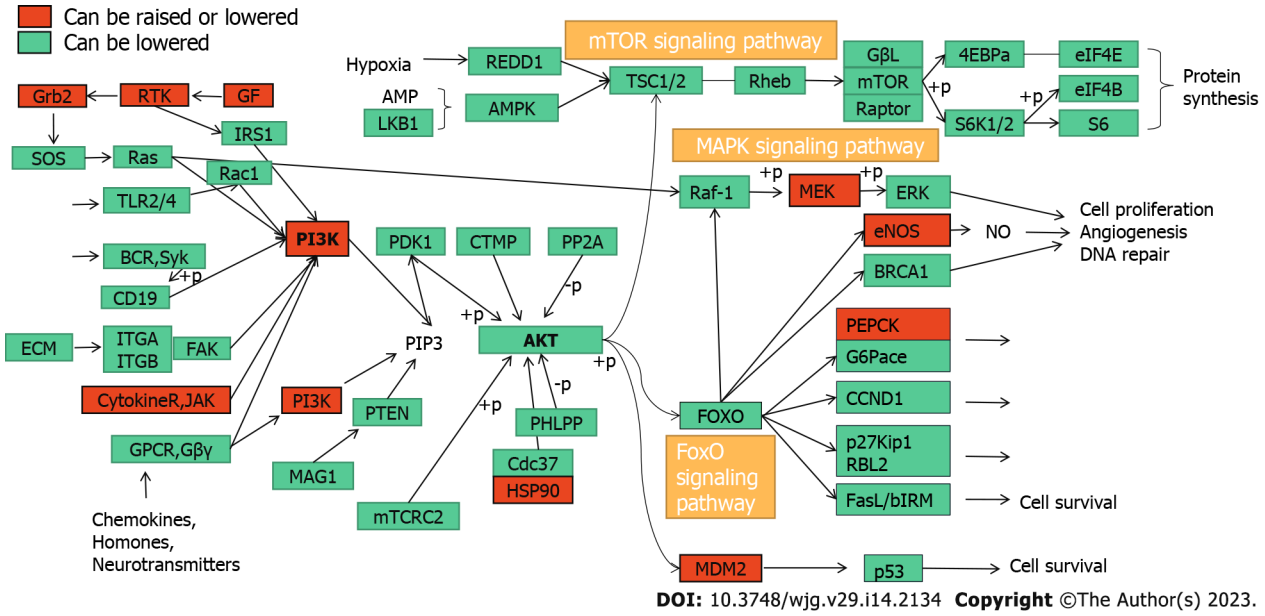
According to the HPLC analysis of the distribution and metabolism of CAM in mice, the maximum concentration of CAM in the intestine was reached 0.5 h after administration and that in the liver, kidney, lung and spleen occurred 2.5 h after administration. CAM was eliminated faster from the liver and intestine than from the kidney, lung and spleen. CAM was not detected at any time point in the brain, indicating that this compounds does not easily cross the blood-brain barrier.

IL-4 is a common inflammatory cytokine that is involved in the occurrence of inflammation, and the inflammatory microenvironment is closely related to the deterioration of tumor cells[5]. IL-4 inhibits the inflammatory response in advanced liver cancer, mainly because an increase in its levels can weaken the extent of the inflammatory response and viral replication in the liver[6]. IL-1β is a proinflammatory cytokine derived from monocytes and macrophages that can participate in the processes of infection,

Table 2 Binding energy between carrimycin and the key target

Key target	Binding energy (kcal/mol)
ALB	-9.3
ESR1	-5.8
EGFR	-6.5
CASP3	-6.1
AR	-5.6
HSP90AA1	-5.0
IGF1	-5.1
MAPK1	-6.7
MAP2K1	-5.2
IL2	-5.8

ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; IGF: Insulin-like growth factor; HSP: Heat shock protein; CASP3: Recombinant caspase 3; AR: Androgen receptor; MAPK1: Mitogen-activated protein kinase 1; IL: Interleukin.

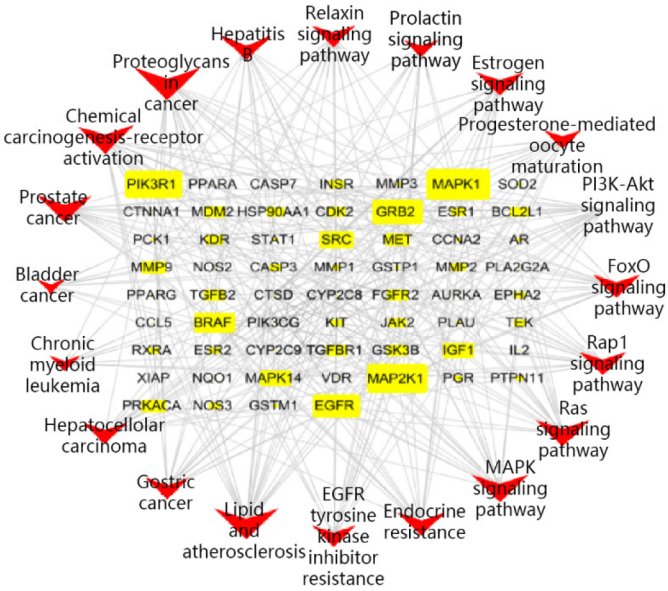


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Figure 8 PI3K-Akt signaling pathway regulation. This plot shows the genes that are upregulated or downregulated (red) and downregulated (green) by carrimycin in the PI3K-Akt pathway. Grb2: Growth factor receptor bound protein 2; RTK: Receptor tyrosine kinase; GF: Growth factor; IRS1: Insulin receptor substrate 1; TLR: Toll-like reCEPT; BCR: Breakpoint cluster region; ECM: Extracellular matrix; ITGA: Integrin subunit alpha; FAK: Focal Adhesion Kinase; JAK: Janus kinase; GPCR: G protein-coupled receptor; AMP: Adenosine Mono Phosphate; LKB1: Liver kinase B1; REDD1: regulated in development and DNA damage responses-1; AMPK: AMP-activated protein kinase; TSC: tuberous sclerosis; Rheb: Right Hand Equipment Bay; PDK1: Pyruvate dehydrogenase kinase, isozyme 1; CTMP: Carboxy terminal modulator protein; PP2A: Protein phosphatase 2A regulatory subunit; PIP: Phosphoinositide-binding protein; PTEN: Phosphatase and tensin homolog; PHLPP: PH domain leucine-rich repeat protein phosphatase; CDC: Cell division cycle gene; HSP: Heat shock protein; RAF: Rheumatoid Arthritis Factor; MEK: Mitogen-activated protein; ERK: Extracellular regulated protein kinases; ENOS: Endothelial nitric oxide synthase; BRCA: Breast cancer susceptibility genes; PEPCK: Phosphoenolpyruvate carboxykinase; FOXO: Forkhead Box O; MDM2: Murine double minute 2; CCND1: Recombinant Cyclin D1; RBL2: Retinoblastoma-like 2; MDM2: Murine double minute 2; mTOR: Mammalian target of rapamycin (a protein).

inflammation, immune injury, tissue destruction and so on[7]. Moreover, relevant studies have shown that IL-1β may be involved in the pathogenesis and evolution of tumors[8]. Therefore, the effects of CAM on inflammatory factors in various tissues and organs were examined. CAM was found in the lung, kidney, liver and spleen and had particularly affected the IL-1β levels in the spleen, liver, and kidney and especially the small intestine and lung.

The network pharmacology results showed that CAM is involved in disease treatment through processes including neutrophil degranulation, neutrophil activation, immune response participation, purine-containing compound metabolism, glycosyl compound metabolism and protein autophos-



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Figure 9 Target-pathway network of carrimycin. The nodes include 60 related targets displayed as yellow rectangles and 20 signaling pathways indicated in red type V. The main targets acting on the pathway include MAPK1, MAP2K1, and EGFR, *etc.*

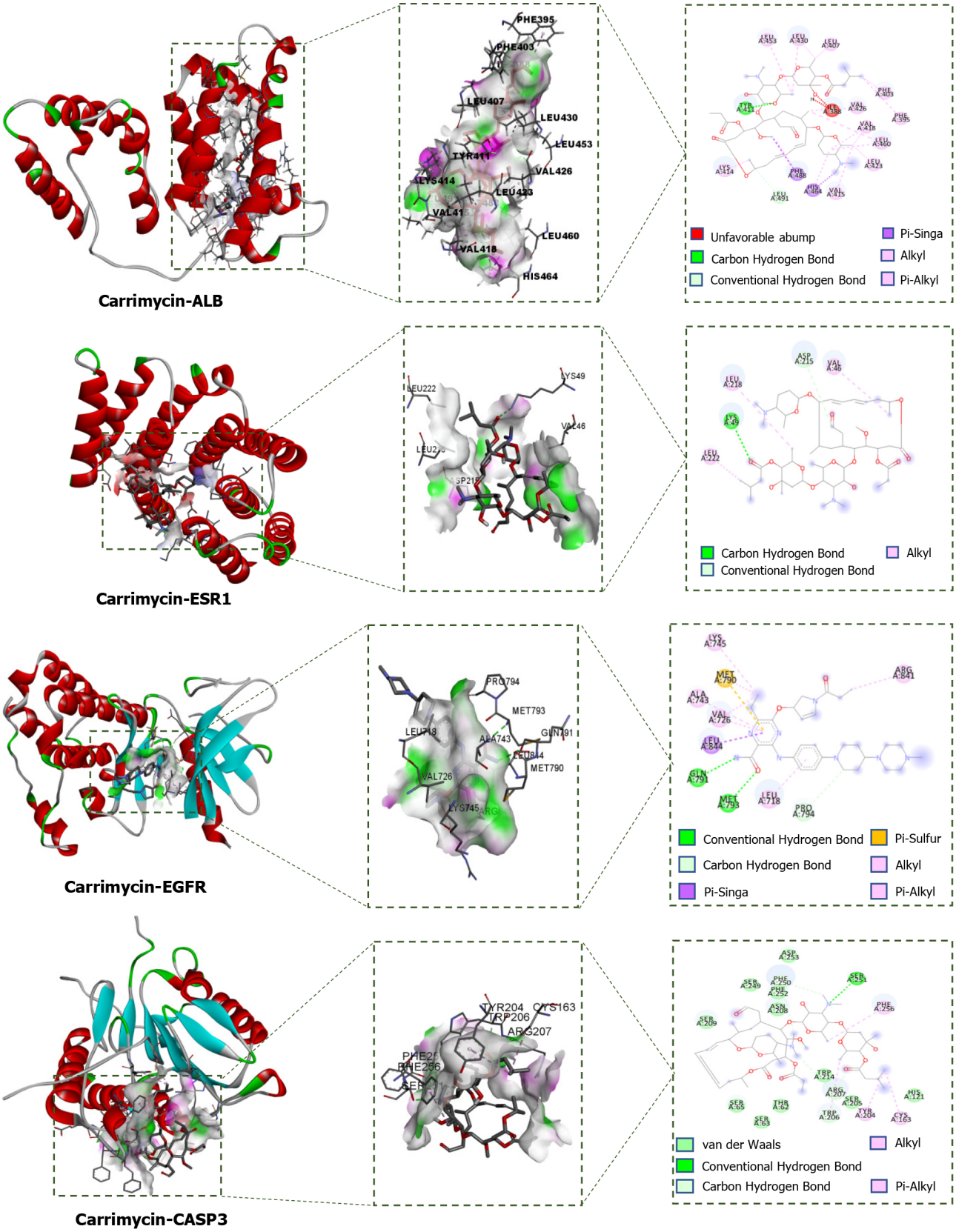
phorylation. Its molecular functions mainly entail carboxylic acid binding, organic acid binding, protein tyrosine kinase activity, endopeptidase activity and serine endopeptidase activity. In terms of cell composition, CAM is mainly involved in vesicle cavity, cytoplasmic vesicle cavity, secretory granule cavity and collagen-containing ECM.

Neutrophils are a kind of natural immune cell, and with the deepening of research, the relationship between neutrophils and tumors has attracted attention. Many studies have shown that neutrophils have potential antitumor functions[9] and cytotoxic effects on tumor cells[10]. Neutrophils are also an important member of the human immune system, playing an important role in the defense mechanisms of the human body[11]. Neutrophils release proteolytic substances such as myeloperoxidase (MPO), neutrophil elastase (NE), and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and active substances with biological effects related to their degranulation functions[12]. MPO and NE are present in the celestial green granules of neutrophils, and their extracellular release *via* degranulation produces biological effects[13]. MPO can catalyze the formation of HOCl *in vivo*, and HOCl and NE can inhibit tumor growth at certain concentrations[14]. TRAIL is a member of the TNF superfamily, whose receptors are expressed and distributed on the surfaces of many tumor cells. Therefore, for some tumor cells, TRAIL can not only induce tumor cell apoptosis but also inhibit tumor angiogenesis through selective killing effects[8].

Purines are heterocyclic aromatic compounds. Since Noell and Robins[15] discovered 2-amino-6-alkyl thiopurine derivatives as antitumor drugs in 1962, considerable efforts have been made to develop purine derivatives with antitumor activity over the last few decades[16]. Purine compounds, such as mercaptopurine, which was discovered earlier, and nelarabine and clofarabine, which were discovered later, have been used as antitumor drugs for many years[17]. Therefore, the metabolic processes of purine-containing compounds have inhibitory effects on tumors.

Protein phosphorylation and dephosphorylation regulate almost all life activities, such as cell signal transduction, differentiation, growth, and apoptosis. Approximately 30% of the proteins in the body can undergo different types of phosphorylation[18]. Abnormal protein phosphorylation often leads to abnormal cellular activities and even cell damage or carcinogenesis[19]. Abnormalities in cellular activities, such as those involved in tumor growth, differentiation, apoptosis and movement, are all accompanied by abnormalities in protein phosphorylation in their corresponding cellular signal transduction pathways.

KEGG analysis of the enriched pathways of the CAM targets showed that the pathways involved in the treatment of diseases mainly included the Ras, FoxO, insulin, estrogen, and peroxisome proliferator-activated receptor (PPAR) signaling pathways. The Ras signaling pathway was named because this protein is a common mediator of the signal transduction processes of many growth factors. It has been reported that the Ras family diabetes-related gene RRAD can inhibit the proliferation, aerobic glycolysis and apoptosis of hepatocellular carcinoma cells by downregulating ACTG1 levels[20]. FoxO transcription factors shuttle through and outside the nucleus to regulate pathways involved in cell differentiation, proliferation, and stress, cell cycle metabolism, and tumor inhibition. The pathogenesis of hepatitis B-related liver cancer is closely related to activation of the FOXM1/FoxO3a signaling pathway[21]. Insulin signaling affects energy metabolism and growth, and this signal is transmitted



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Figure 10 Molecular docking poses of the core targets in liver cancer. In each of the docking simulations, hydrogen bonds were found between carrimycin and the four targets. The binding energies were all under -5, which suggested that the docking results were good and that the drug could bind to the target. ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; CASP3: Recombinant caspase 3.

through the Akt branch. The Akt signaling pathway can inhibit tumor cell proliferation, induce apoptosis and downregulate the expression of Bcl-2[22]. Akt is a regulatory molecule downstream of PI3K. Akt is activated after being phosphorylated and can affect the expression of the downstream

metastasis-related gene MMP-9, the apoptosis-related gene Bcl-2 and the proliferation-related gene cyclin D1[23]. Akt plays an important role in regulating cell proliferation, metastasis and apoptotic processes, and its hyperactivation is closely related to the development of various tumors, including liver cancer[24]. Dysregulation of the estrogen signaling pathway is an important factor in the development of many malignant tumors, such as those found in breast cancer, liver cancer and lung cancer. Estrogen exerts its physiological and pathological functions by binding to the estrogen receptor (ESR). ESR-α36 expression is upregulated in hepatocellular carcinoma, so ESR-α36 may be involved in the rapid estrogen signal transduction that mediates hepatocarcinogenesis[25]. Abnormal expression of ESR-α36 in the liver is related to hepatocyte proliferation. Estrogen passes through the ESR-α36 and EGFR/SRC axes to induce MAPK/ESR-K phosphorylation and promote the expression of cyclin D1. ESR-α36 interacts with the EGFR/SRC/SHC complex through the EGFR/ESR-α36/ERK signaling axis and mediates rapid estrogen signal transduction in hepatoma cells. Thus, the ESR-α36/EGFR signaling loop is a potential target for the development of a new approach to treat liver cancer, as the ESR-α36/EGFR positive regulatory loop plays an important role in the maintenance and positive regulation of hepatoma cells[26]. PPAR-γ is an extremely important regulator of fat and glucose metabolism and plays an important role in autoimmune responses and inflammatory processes[27]. PPAR-γ is expressed in bronchial airway epithelial cells in situ and can inhibit the activity of these cells, activate endogenous PPAR-γ, and antagonize the inflammatory response. Zhu *et al*[28] suggested that PPAR-γ can affect the transport of macrophages and increase their phagocytosis, promoting the occurrence of a practical immune response. PPAR-γ ligand stimulation in tumor cells and tissues can have a major antitumor effect[29].

According to the analysis of CAM targets, EGFR intersects with multiple signaling pathways, including those in healthy tissues and liver cancer, breast cancer, kidney cancer, colon cancer and other tumor cells[30]. When EGFR binds to its ligand, the receptor can dimerize specific tyrosine residues can be phosphorylated. These phosphorylated tyrosine residues can recruit other signaling molecules to initiate the downstream signal transduction pathway that mediates the proliferation, metastasis and drug resistance of tumor cells. In liver cancer tissues, the expression of EGFR is significantly upregulated. For example, when EGFR is activated by its ligand, it can mediate the growth, proliferation and metastasis of liver cancer cells through multiple signaling pathways, such as the PI3K-Akt, JAK/STAT3, and Ras/MEK/ERK pathways[31]. In organisms, a variety of proteases participate in the degradation of the basement membrane and ECM, among which MMPs play the most prominent role. MMPs are the main targets of drugs against tumor invasion and metastasis. MMP-2 can specifically degrade the main components of the basement membrane and intercellular matrix (type IV collagen) and improve tumor cell invasiveness and metastasis. Therefore, MMP-2 is considered one of the most important MMPs in these processes[32]. It has been reported that a low level of MMP-2 expression can promote both the activation of nuclear factor-kappa B protein signaling and the invasion and metastasis of lung cancer[33]. MMP-3 is one of the key enzymes involved in ECM synthesis and the regulation of catabolic activity. MMP-3 is also closely related to the invasion and metastasis of tumor cells[34].

After analysis of the PPI network constructed with the intersecting targets of CAM and liver cancer, it was found that ALB, IGF1, ESR1, EGFR, AR, MAPK1, SRC, MAPK14, KIT, MMP-2, and CASP3 are both involved in the pathological process of liver cancer and also key targets of CAM. Molecular docking showed that CAM could bind well to ALB, ESR1, EGFR, CASP3 and other targets through hydrogen, conjugated and other bonding interactions, which further confirmed the therapeutic effect of CAM on liver cancer. Studies have shown that ALB is a serum protein that is almost exclusively expressed in liver tissues and that ALB genes have a closed conformation in other tissues[35]. Dai *et al*[36] showed that ESR1 expression is a candidate tumor suppressor gene in hepatocellular carcinoma (HCC). Further, promoter hypermethylation may be a mechanism by which expression of ESR1 is repressed, and the extent of hypermethylation of ESR1 may be a marker for HCC status and progression. EGFR is at the intersection of multiple signaling pathways and also an important mediator of physiological activities such as cell growth, proliferation, differentiation and adhesion. EGFR expression levels were found to be low in healthy tissues but significantly high in liver, breast, kidney, colon and other tumor cells[30]. When EGFR binds to its ligand, the receptor can dimerize specific tyrosine residues can be phosphorylated. These phosphorylated tyrosine residues can recruit other signaling molecules to initiate the downstream signal transduction pathway that mediates the proliferation, metastasis and drug resistance of tumor cells. The expression of EGFR was found to be significantly upregulated in hepatocellular carcinoma, and high EGFR expression is also closely related to the malignant transformation of hepatocellular carcinoma[37]. A large number of studies have reported the role of EGFR in the occurrence and development of liver cancer. For example, when EGFR is activated by its ligand, it can mediate the growth, proliferation and metastasis of liver cancer cells through multiple signaling pathways, such as the PI3K-Akt, JAK/STAT3, and Ras/MEK/ERK pathways[31]. CASP3 is a protease that can specifically cleave poly ADP ribose polymerase and acetyl-devd-7-amino-4-methyl-coumarin, resulting in DNA cleavage and the promotion of apoptosis, both of which play a core roles in the executive stage of apoptosis. Xie *et al*[38] have confirmed activation of macrophages plays a critical role in liver injury and fibrogenesis during hepatitis B virus (HBV) infection. And hepatitis Be antigen (HBeAg) can obviously induce the production of macrophage inflammatory cytokines. During HBV-induced macrophage activation, the ESR-K/CREB signaling pathway promotes miR-212-3p expression.

MiR-212-3p can inhibit the production of inflammatory cytokines induced by HBeAg by regulating the target gene MAPK1.

According to GO analysis of the intersecting genes of CAM and liver cancer targets, CAM was found to be involved in the regulation of the inflammatory response and protein kinase B signaling associated with cancer. KEGG analysis of the effects of CAM on the key targets of liver cancer showed that many signaling pathways, such as the FoxO, PI3K-Akt, estrogen, Ras, and MAPK signaling pathways, coincide with the pathways of the predicted targets of CAM, which proves that CAM can indeed affect liver cancer cells through the activities of multiple targets and multiple pathways.

CONCLUSION

In this study, we examined the distribution and metabolism of CAM and its effects on inflammatory factors in various tissues and organs of mice. Using reverse molecular docking technology, we screened for diseases related to CAM activity in liver cancer. Moreover, we systematically analyzed the targets and biological pathways of CAM in the treatment of liver cancer by network pharmacology and molecular docking to further explore the mechanism by which CAM is involved in disease treatment. However, network pharmacology methods are based on data research, so there are some remaining limitations, such as incomplete database data, imperfect screening criteria for components and targets and diverse pathway enrichment methods, which will affect the accuracy of the research results. Therefore, follow-up experiments need to be carried out on the material basis of the pharmacodynamic pathways to verify the research results here, which can provide a theoretical and experimental basis for treating liver cancer or inflammation with CAM.

ARTICLE HIGHLIGHTS

Research background

As a new national first-class drug, carrimycin (CAM) has much potential medicinal value. The clinical results show that its antibacterial activity and pharmacodynamic activity are significantly greater than those of similar antibiotics. Through the combination of animal experiments and network pharmacology, this study demonstrates that CAM plays a positive role in the treatment of inflammation and some cancers.

Research motivation

At present, the mechanism of CAM in the treatment of liver cancer is not clear, so the mechanism can be speculated by network pharmacology.

Research objectives

To gain a deeper understanding of CAM, the distribution, metabolism and anti-inflammatory effects of CAM in organs were assessed, and the anti-liver cancer mechanism of CAM was analyzed.

Research methods

Taking the content of isovaleryl spiramycin III as the index, the distribution and metabolism of CAM in various tissues and organs of mice and its effect on inflammatory factors in various tissues and organs of mice were detected. The target of CAM was determined by reverse molecular docking technology, the disease type corresponding to each target protein was selected, the target protein disease type network was established, and the key targets of CAM in liver cancer were screened by network pharmacological methods. The core target was verified by molecular docking and visual analysis.

Research results

The maximum CAM concentration was reached in the liver, kidney, lung and spleen 2.5 h after intragastric administration. In the intestine, the maximum drug concentration was reached 0.5 h after administration. CAM is predicted to regulate related pathways by acting on many targets, such as albumin, estrogen receptor 1, epidermal growth factor receptor and caspase 3, to treat cancer, inflammation and other diseases.

Research conclusions

CAM had an inhibitory effect on inflammation. It also predicts the multitarget, complexity of CAM involving multiple pathways and the diversity of CAM effects in the treatment of liver cancer, which provides a basis and direction for further clinical research.

Research perspectives

We examined the distribution and metabolism of CAM in murine tissues and organs and the effect of CAM on inflammatory factors and performed a systematic analysis of the targets and biological pathways involved in the treatment of liver cancer with CAM by network pharmacology, which led to further insights into the mechanism of treatment of the disease with CAM.

FOOTNOTES

Author contributions: Li XY, Luo YT, Guan QX, Wang YH, Yang ZX, and Shang YZ performed the experiments and acquired and analyzed the data; Li XY and Luo YT wrote the manuscript; and all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Effect and mechanism of reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3 inflammasome activation in hepatic alveolar echinococcosis

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Abstract

BACKGROUND

The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is a significant component of the innate immune system that plays a vital role in the development of various parasitic diseases. However, its role in hepatic alveolar echinococcosis (HAE) remains unclear.

AIM

To investigate the NLRP3 inflammasome and its mechanism of activation in HAE.

METHODS

We assessed the expression of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE. A rat model of HAE was employed to investigate the role of the NLRP3 inflammasome in the marginal zone of HAE. Transwell experiments were conducted to investigate the effect of *Echinococcus multilocularis* (*E. multilocularis*) in stimulating Kupffer cells and hepatocytes. Furthermore, immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay were used to evaluate NLRP3, caspase-1, IL-1β, and IL-18 expression; flow cytometry was used to detect apoptosis and reactive oxygen species (ROS).

RESULTS
NLRP3 inflammasome activation was significantly associated with ROS. Inhibition of ROS production decreased NLRP3-caspase-1-IL-1β pathway activation and mitigated hepatocyte damage and inflammation.

CONCLUSION
E. multilocularis induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, indicating that ROS may serve as a potential target for the treatment of HAE.

Key Words: Hepatic alveolar echinococcosis; Inflammasome; Inflammation; Kupffer cell; NLR family pyrin domain-containing 3 protein; Reactive oxygen species

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Core Tip: In recent years, the role of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in parasitic diseases has attracted widespread attention. However, the role and clinical significance of the NLRP3 inflammasome in Hepatic alveolar echinococcosis (HAE) remain unclear. Herein, we explored the mode of NLRP3 inflammasome activation in the tissues of patients with HAE, a rat model of HAE, rat Kupffer cells, and hepatocytes. Our experiments showed that inhibiting reactive oxygen species (ROS) production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation and apoptosis rates in hepatocytes in HAE. We conclude that ROS-mediated NLRP3 inflammasome activation was a key factor leading to hepatocyte injury and triggering a cascade of inflammatory reactions. Therefore, ROS production may be a promising target for the treatment of HAE.

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INTRODUCTION

Echinococcosis is a global zoonotic parasitic disease categorised into two main subtypes, namely cystic echinococcosis and alveolar echinococcosis, which are caused by the larvae of *Echinococcus granulosus* and *Echinococcus multilocularis* (*E. multilocularis*), respectively. Alveolar echinococcosis, also termed "parasite cancer"[1], is a helminthiasis that progresses slowly and mainly invades the liver. However, if left untreated, it can lead to high morbidity and mortality[2]. It is estimated that nearly 600000 people worldwide have been infected[3,4]. Cases of hepatic alveolar echinococcosis (HAE) have risen sharply in recent years. Qinghai Province in China is a region with a high and increasing incidence of echinococcosis[5]. Moreover, epidemiological evidence suggests that the medical and economic burden of HAE will increase significantly in the next decade[6].

Inflammasomes were first proposed by Martinon *et al*[7] in 2002 as multimeric complexes that form in response to various physiological and pathological stimuli. Inflammasomes are important components of the innate immune system, and inflammasome activation is essential for pathogen clearance[8]. The main components include nucleotide-binding oligomerization domain-like receptors (NLRs), leucine-rich repeats, and a protein-protein interaction domain, which could be a pyrin domain, a caspase recruitment domain, or a baculovirus inhibitor of an apoptosis protein repeat domain. NLRs are cytosolic pattern-recognition receptors that act as sensors of the innate immune system. They can recognize microbial structures or pathogenic components, called pathogen-associated molecular patterns or damage-associated molecular patterns, which are generated by endogenous stress, and trigger downstream inflammatory pathways, to eliminate infection and repair damaged tissues[9]. An inflammasome is defined by its sensor protein. Five pattern-recognition receptors have been shown to form inflammasomes: Absent in melanoma 2 (AIM2), NLR family caspase recruitment domain-containing 4 (NLRC4), NOD-like receptor family pyrin domain-containing 1 (NLRP1), NLRP3, and pyrin[10]. Among them, the NLRP3 inflammasome is the most studied.

The NLRP3 inflammasome, a signaling molecule in the innate immune system, can convert inactive cysteine aspartate proteolytic enzyme 1 precursor (pro-caspase-1) into active caspase-1, which in turn

converts inactive pro-interleukin (IL)-1β and pro-IL-18 into mature IL-1β and IL-18, respectively. NLRP3 inflammasome expression is transcriptionally regulated by nuclear factor kappa B (NF-κB), and is very low in non-activated macrophages[11]. NLRP3-caspase-1-IL-1β pathway activation exerts effects on processes, such as stress, inflammation, and injury repair[12], which have been extensively studied in tumour development[13-15] and metastasis[16]. In recent years, the role of the NLRP3 inflammasome in parasitic diseases has attracted widespread attention. The NLRP3 inflammasome is upregulated in malaria[17]. Furthermore, in amoebic diseases, macrophage α5β1 integrin expression is associated with NLRP3 inflammasome activation[18]. The NLRP3 inflammasome and caspase-1/11 pathway protect against acute *Trypanosoma cruzi* (*T. cruzi*) infection[19]. The NLRP3 inflammasome controls *T. cruzi* infection *via* a caspase-1-dependent, IL-1R-independent, nitric oxide mechanism[20]. Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to *Leishmania*[21]. The development of Leishmaniasis occurs *via* NLRP3 inflammasome-mediated IL-1β production[22]. *Schistosoma mansoni* activates the NLRP3 inflammasome and alters adaptive immune responses through Dectin-2[23]. NLRP3 inflammasome activation in mice results in fibrosis of hepatic stellate cells in schistosomiasis [24]. Although the NLRP3 inflammasome is activated differently in different parasitic diseases, it plays an important role in these diseases. Assessing inflammasomes may aid in the rapid identification and elimination of these pathogenic factors, and provide potential means for the treatment and prevention of infection.

NLRP3 inflammasome activation is related to mitochondrial autophagy and reactive oxygen species (ROS) production[25,26]. The mechanism of ROS-mediated NLRP3 inflammasome activation is closely related to Kupffer cells[27,28]. Inhibiting ROS production or using nicotinamide adenine dinucleotide phosphate, an oxidase inhibitor, can block NLRP3 inflammasome activation[29,30]. As an important mediator of oxidative stress, ROS is closely related to inflammation. *T. cruzi* infection promotes the synthesis and release of ROS, with NF-κB pathway activation and decreased expression of inflammatory cytokines in NLRP3 knock-out mice, while alleviating acute-phase symptoms[31]. Protein kinase C/ROS-mediated NLRP3 inflammasome activation correlates with pathological changes in leishmaniasis [32]. ROS inhibition and prevention of potassium channel opening inhibits NLRP3 inflammasome expression, implying that ROS production and potassium channel opening are pivotal for NLRP3 inflammasome activation in human prostate epithelial cells[33].

The occurrence and development of parasitic diseases is often accompanied by inflammation[34,35]. In a previous study, we determined that HAE has a clear marginal zone[36]. However, the role and clinical significance of the NLRP3 inflammasome in HAE remain unclear. Herein, we explored NLRP3 inflammasome activation in the tissues of patients with HAE, a rat model of HAE, rat Kupffer cells, and hepatocytes. We investigated the mechanism of NLRP3 inflammasome activation in HAE and the effect of its downstream products. We hypothesised that *E. multilocularis* aggravates hepatic damage and inflammation by activating the NLRP3 inflammasome in HAE.

MATERIALS AND METHODS

Patient tissues

Tissue specimens were collected from 60 patients with HAE who underwent surgery at the Department of Hepatobiliary and Pancreatic Surgery of the Affiliated Hospital of Qinghai University from January to December 2020. Patients did not receive adjuvant therapy before surgery. HAE was confirmed by pathological examination. The pathological stage and degree of differentiation of the lesions were based on the PNM classification, where P refers to a parasitic mass in the liver, N refers to the involvement of neighbouring organs, and M refers to metastasis[37]. The marginal zone of the lesion (< 0.5 cm away from the lesion) and the tissue adjacent to the lesion (> 3 cm away from the lesion) were rapidly frozen in liquid nitrogen, within 15 min of surgical resection, and stored at -80 °C.

Parasites and animal experiments

E. multilocularis specimens were obtained from the Key Laboratory of Echinococcosis, Qinghai Province, China, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Burlington, Ontario, Canada) supplemented with 10% foetal bovine serum (FBS) (Gibco) at 37 °C in 5% carbon dioxide. Sprague-Dawley rats were purchased from the Nanjing Qinglongshan Laboratory Animal Breeding Centre, China. Feeding and housing were performed under specific pathogen-free conditions. Eight- to 10-wk-old male Sprague-Dawley rats were used as hosts. Rats were fixed in the dorsal position and anaesthetised with 1%-3% isoflurane. The abdominal cavity was opened, and the skin, superficial fascia, deep fascia, muscle, and peritoneum were dissected to expose the liver. A syringe needle was inserted into the liver obliquely at a 45-degree angle (penetration depth, 0.5 cm). Each rat was injected with a suspension of approximately 1200-1500 *E. multilocularis*. The method of counting the number of *E. multilocularis* was similar to the cell counting plate method. After injecting the suspension, the liver was covered with absorbable hemostat gauze to stop bleeding, and the abdominal incision was closed. For *in vivo* experiments, N-tert-Butyl-α-phenylnitrone (PBN; Sigma, Saint Louis, MO, United States) was used as a ROS scavenger. Nine 6-mo-old HAE rats were divided into three groups (three rats per group), to

test the efficacy of PBN[38,39]. The three groups were treated with different concentrations-20, 50, and 100 mg/kg/d-intraperitoneally for 30 d. ROS production in the three groups was used to determine the relative efficient concentration of PBN in HAE rats. Subsequently, 30 HAE rats were randomly divided into three groups (10 rats per group), namely the 50 mg/kg/d PBN group, normal saline group, and no-treatment group (control). Intraperitoneal injection was performed at the same time and the same dose (50 mg/kg/d) in the PBN and normal saline groups for 30 d.

Immunohistochemistry and haematoxylin eosin staining

Paraffin sections were incubated in 3% hydrogen peroxide at 25 °C for 5-10 min to inhibit endogenous peroxidase activity. Primary antibodies (NLRP3: Abcam, Shanghai, China; caspase-1: Thermo Scientific, Waltham, MA, United States; and IL-1β and IL-18: Boster Biotechnology, Wuhan, China) were added and incubated at 4 °C overnight, washed with phosphate-buffered saline (PBS), and incubated with the appropriate secondary antibody at 20-37 °C for 10-30 min. Haematoxylin and eosin staining was performed, as described previously[40]. The optical density and area of each image were measured using an Image-Pro Plus 6.0 Image Analysis System (Media Cybernetics Inc., Bethesda, MD, United States), and the mean density was calculated.

Immunofluorescence staining for cell localisation

Normal goat serum blocking solution was added to reduce background staining. Primary antibodies (CD68: Proteintech, Wuhan, China; NLRP3: Abcam; and caspase-1: Thermo Scientific) were added. The sections were incubated at 4 °C overnight, washed with PBS, and incubated with fluorescent secondary antibodies for 2 h, followed by a 30-min incubation in the dark with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, Shanghai, China). Cells were observed and images captured under a fluorescence microscope (Olympus BX33; Olympus Corp., Tokyo, Japan).

Analysis of ROS production and apoptosis by flow cytometry

To detect ROS, Kupffer cells were resuspended in 2′-7′-dichlorofluorescin diacetate from a ROS Assay Kit (Nanjin Jiancheng Bioengineering Institute, Nanjing, China) and incubated at 25 °C for 30 min. The liver was cut into 2-3 mm^3 sections with scissors and single-cell suspensions were harvested for ROS detection. Cells were washed twice with PBS. Relative ROS production was detected through the fluorescein isothiocyanate (FITC) channel (500 nm). For the apoptosis assay, cells were stained with Annexin V-FITC (MultiSciences, Hangzhou, China) and propidium iodide, according to the manufacturer's instructions. Briefly, 1-10 × 10^5 cells were washed with PBS and resuspended in 500 μL of 1 × binding buffer. Next, 5 μL Annexin V-FITC and 10 μL propidium iodide were added to each tube. The cells were incubated at 25 °C in the dark for 5 min. Flow cytometry was performed on a FACSCalibur (Becton and Dickinson, Rutherford, NJ, United States). FlowJo software version 10.5 (Becton and Dickinson) was used to analyse the data.

Co-cultivation experiments

For the co-cultivation experiments, normal Buffalo rat liver cells were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences, Shanghai, China. Buffalo rat liver cells (3 × 10^5) were plated in the top chamber of a 6-well plate (0.4-μm Pore Polycarbonate Membrane Insert; Corning Life Sciences, Shanghai, China). *E. multilocularis* (1 × 10^3) and rat liver macrophages (1 × 10^6) were cultured in the lower chamber. Buffalo rat liver hepatocytes, liver macrophages, and *E. multilocularis* were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco). N-acetyl-L-cysteine (NAC; Macklin Biochemical, Shanghai, China) was chosen as a ROS scavenger[41] in a co-culture system, by treating cells with 5, 10, or 20 mmol/L NAC and detecting ROS production in Kupffer cells at 24, 48, and 72 h, to test its efficacy. The supernatant was collected and stored at -80 °C. IL-1β and IL-18 were detected, along with NLRP3, caspase-1, and ROS.

Western blotting

Protein samples were quantified using a BCA Protein Assay Kit (Bio-Rad, Mississauga, Ontario, Canada). Western blotting was performed, as described previously[42]. Briefly, samples were subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to polyvinylidene fluoride membranes, incubated with primary antibodies (NLRP3: Abcam; and caspase-1: Thermo Scientific) overnight at 4 °C, washed, and blotted with the Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (Abcam). The blots were developed with ECL reagent (Sangon Biotech, Shanghai, China) using an Image Quant LAS 4000 Imaging System (GE Healthcare, Chicago, IL, United States) and analysed using Image-Pro Plus 6.0 (Media Cybernetics Inc.). Band intensity was normalised to the intensity of b-actin (Abcam).

Enzyme-linked immunosorbent assay

The Rat IL-1b ELISA Kit (Abcam) and Rat IL-18 enzyme-linked immunosorbent assay (ELISA) Kit (MultiSciences) were used, according to the manufacturers' instructions, to determine IL-1b and IL-18 levels in serum and the co-cultured cell supernatant. Each sample was assayed in duplicate and the

optical density of each well was measured immediately using a microplate reader (iMark; Bio-Rad, Hercules, CA, United States).

Extraction and identification of Sprague-Dawley rat Kupffer cells

Kupffer cells were isolated from Sprague-Dawley rats by collagenase perfusion and density gradient centrifugation[43,44]. Briefly, Sprague-Dawley rat liver tissue was perfused with type IV collagenase (Beyotime Biotechnology) and cut into 2-3 mm^3. The cell suspension was separated by centrifugation at 500 × g for 5 min, followed by the addition of 30% Percoll density gradient separation solution (Macklin Biochemical) and centrifugation at 900 × g for 15 min. Kupffer cells were collected from the interface between the PBS and 30% Percoll layers and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin at 37 °C in 5% carbon dioxide. For identification, cells were adhered to a glass coverslip, fixed with 4% paraformaldehyde for 15 min, and blocked with normal goat serum. Diluted (1:100) primary antibody (CD68: Proteintech) was added and incubated at 4 °C overnight, followed by incubation with fluorescent secondary antibody at 20-37 °C for 1 h. DAPI (Beyotime Biotechnology) was added, with a further incubation in the dark for 5 min. Cells were observed and images captured under a fluorescence microscope (Olympus BX33; Olympus Corp.) at 200 × and 400 × magnification.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, United States). The Pearson test was used for correlation analysis and the chi-squared test was applied to analyze the relationship between NLRP3 expression and the clinicopathological characteristics of patients with HAE. Quantitative data are presented as the mean ± SD. Data were analyzed using student's *t*-test or one-way analysis of variance, as appropriate. All *P* values were two-sided; as $P < 0.05$ was considered statistically significant.

RESULTS

NLRP3, Caspase-1, and IL-1β were upregulated in the marginal zone and NLRP3 expression was associated with HAE

Hematoxylin and eosin-stained tissues from patients with HAE exhibited a clear marginal zone with inflammation (Figure 1A). Immunohistochemical staining of NLRP3, caspase-1, IL-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE (Figure 1B) showed that the expression of NLRP3, caspase-1, and IL-1β was higher in the marginal zone than in the corresponding normal liver, with no significant difference in IL-18 expression (Figure 1C). Using the median expression level of NLRP3 (0.45) as the cut-off, the 60 patients with HAE were divided into a high expression group ($n = 30$) and a low expression group ($n = 30$). Clinicopathological analysis showed that NLRP3 expression in the marginal zone of patients with HAE was associated with jaundice symptoms and Child-Pugh class, but not age, sex, alpha-fetoprotein, or primary lesion (Table 1).

Western blotting (Figure 1D and E) also showed that the expression of NLRP3 (Figure 1F) and caspase-1 (Figure 1G) was higher in the marginal zone than in the corresponding normal liver. These results suggest that the NLRP3-caspase-1-IL-1β pathway may be activated in HAE and that this activation may play a role in the marginal zone.

ROS production increased in the marginal zone and was associated with NLRP3 activation

Lesion growth in HAE rats is shown in Figure 2A. Higher ROS production was observed in the marginal zone compared to the corresponding normal liver. Similarly, cells in the marginal zone of HAE liver showed higher ROS production (Figure 2B and C). ROS production correlated with the relative levels of NLRP3 (linear correlation co-efficient, $r = 0.9489$) (Figure 2D). Thus, we speculated that ROS production may play a role in NLRP3-caspase-1-IL-1β pathway activation in the marginal zone of HAE.

ROS-mediated NLRP3 inflammasome activation in HAE rats

PBN (50 mg/kg/d) significantly reduced ROS production (Figure 3A) and was used in subsequent experiments. After 30 d of intervention, ROS production was detected in the marginal zone of HAE rats in each group (Figure 3B). The relative values of the marginal zone are shown in Figure 3C. Immunohistochemical staining of NLRP3, caspase-1, IL-1β, and IL-18 in the HAE rat marginal zone and corresponding normal liver, after 30-days of treatment, is shown in Figure 3D. The expression of NLRP3, caspase-1), and IL-1β was higher in the marginal zone than in the corresponding normal liver, with no significant difference in IL-18 expression (Figure 3E). NLRP3 and caspase-1 protein expression in the marginal zone and corresponding normal liver is shown in Figure 3F; their relative protein levels are shown in Figure 3G and H, respectively. The Pearson test showed a correlation between ROS production and NLRP3 expression (Figure 3I), and between NLRP3 expression and inflammation in the marginal zone (Figure 3J).

Table 1 Correlation between NOD-like receptor family pyrin domain-containing 3 expression and clinicopathological parameters

Characteristics	*n*	NLRP3		*P* value
		Low expression, *n* = 30	High expression, *n* = 30	
Age (yr)				0.787
≥ 50	21	11	10	
< 50	39	19	20	
Sex				0.195
Male	33	14	19	
Female	27	16	11	
Serum AFP (ng/mL)				0.796
< 20	32	16	15	
≥ 20	28	14	15	
Jaundice symptoms				< 0.001
Yes	34	8	26	
No	26	22	4	
Primary lesion size (cm)				0.071
≥ 5	51	23	28	
< 5	9	7	2	
Child-Pugh class				0.012
A	42	26	16	
B	18	4	12	

AFP: Alpha-fetoprotein; NLRP3: NOD-like receptor family pyrin domain-containing 3.

E. multilocularis activated the NLRP3-caspase-1-IL-1β pathway in Kupffer cells

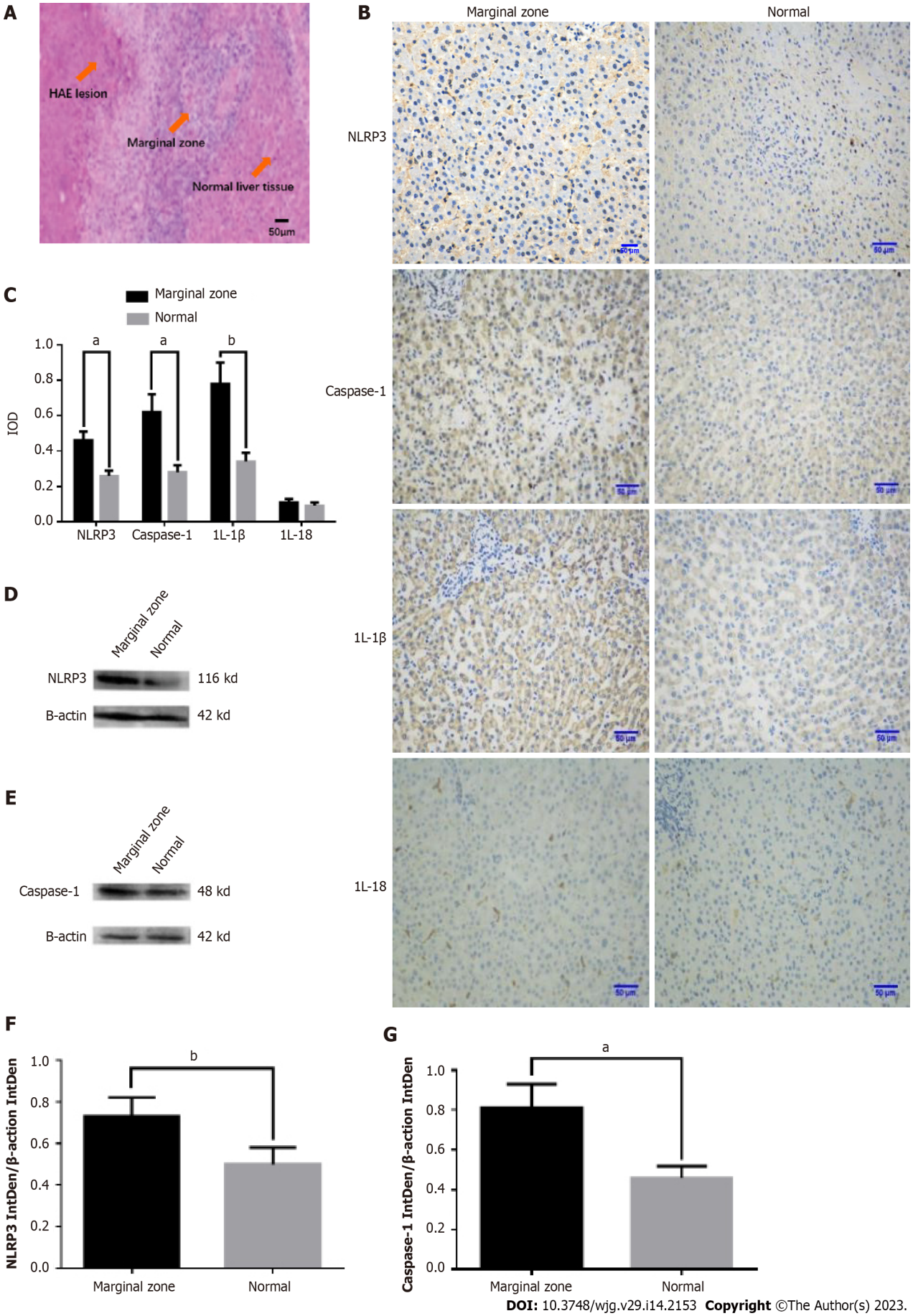
Cells expressing NLRP3 and caspase-1 were localized in the marginal zone of HAE rats (Figure 4A and B). This suggests that NLRP3 and caspase-1 are highly expressed in macrophages (Kupffer cells), with no obvious expression in hepatocytes. To test whether *E. multilocularis* activates the NLRP3-caspase-1-IL-1β pathway in rat Kupffer cells by enhancing ROS production, which promotes IL-1β synthesis and release, leading to hepatocyte damage and triggering an acute inflammatory response, Kupffer cells were isolated (Figure 4C and D). *E. multilocularis* was also isolated from HAE lesions (Figure 4E). In 24-h co-cultures of *E. multilocularis* and Kupffer cells, ROS production was significantly increased in Kupffer cells (Figure 4F).

ROS-mediated NLRP3-Caspase-1-IL-1β pathway activation in Kupffer cells

NAC (5 mmol/L) effectively abrogated ROS production in co-cultured cells (Figure 5A). Co-cultures of *E. multilocularis*, Kupffer cells, and hepatocytes were prepared (Figure 5B). ROS production in Kupffer cells at 24 h, 48 h, and 72 h with or without NAC is shown in Figure 5C. Significant suppression of ROS production was evident in NAC-treated co-cultures (Figure 5D). The effects on IL-1β and IL-18 expression are shown in Figure 5E and F. Hepatocyte apoptosis at 24 h, 48 h, and 72 h is shown in Figure 5G, with relative levels shown in Figure 5H. Western blotting analysis of NLRP3 and caspase-1 is shown in Figure 5I, NLRP3 IntDen/β-actin IntDen protein expression in Figure 5J, and caspase-1 IntDen/β-actin IntDen protein expression in Figure 5K. The apoptosis rate and expression of NLRP3 and caspase-1 were significantly reduced after inhibiting ROS production.

DISCUSSION

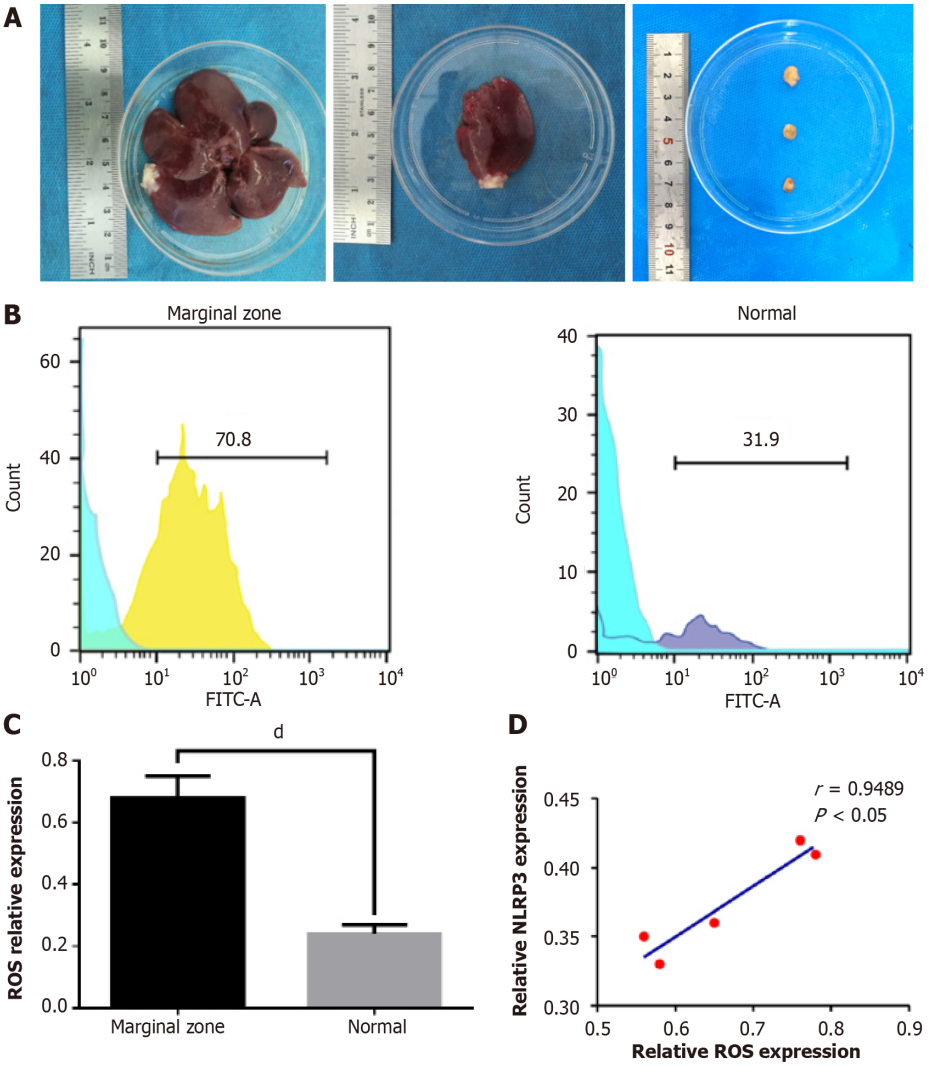
As a global zoonotic parasitic disease, the occurrence of HAE is rare; however, if left untreated, it can lead to high morbidity and mortality, with significant economic burden. Inflammation is crucial in the pathogenesis and progression of serious liver diseases[45-47]. Activation of the inflammatory response is inextricably linked to the activation of innate immunity[12]. The NLRP3 inflammasome is a critical component of the innate immune system that mediates the secretion of pro-inflammatory cytokines in



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Figure 1 NOD-like receptor family pyrin domain-containing 3, caspase-1, and interleukin-1β were upregulated in the marginal zone and

NOD-like receptor family pyrin domain-containing 3 expression was associated with hepatic alveolar echinococcosis. A: Hematoxylin and eosin staining of the lesion, marginal zone, and corresponding normal liver in hepatic alveolar echinococcosis; B: Immunohistochemical staining of NOD-like receptor family pyrin domain-containing 3 (NLRP3), caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver; C: Relative expression of NLRP3, caspase-1, IL-1β, and IL-18; D: NLRP3 protein expression evaluated by western blotting; E: Caspase-1 protein expression evaluated by Western blotting; F: Relative protein expression levels of NLRP3 IntDen/β-actin IntDen; G: Relative protein expression levels of caspase-1 IntDen/β-actin IntDen. C, n = 60. Scale bar: 50 μm. Western blotting was performed in triplicate (mean ± SD). $^{a}P < 0.05$; $^{b}P < 0.01$. HAE: Hepatic alveolar echinococcosis; IOD: Integral Optical Density; NLRP3: NOD-like receptor family pyrin domain-containing 3. IL-1β: Interleukin-1β; IL-18: Interleukin-18.

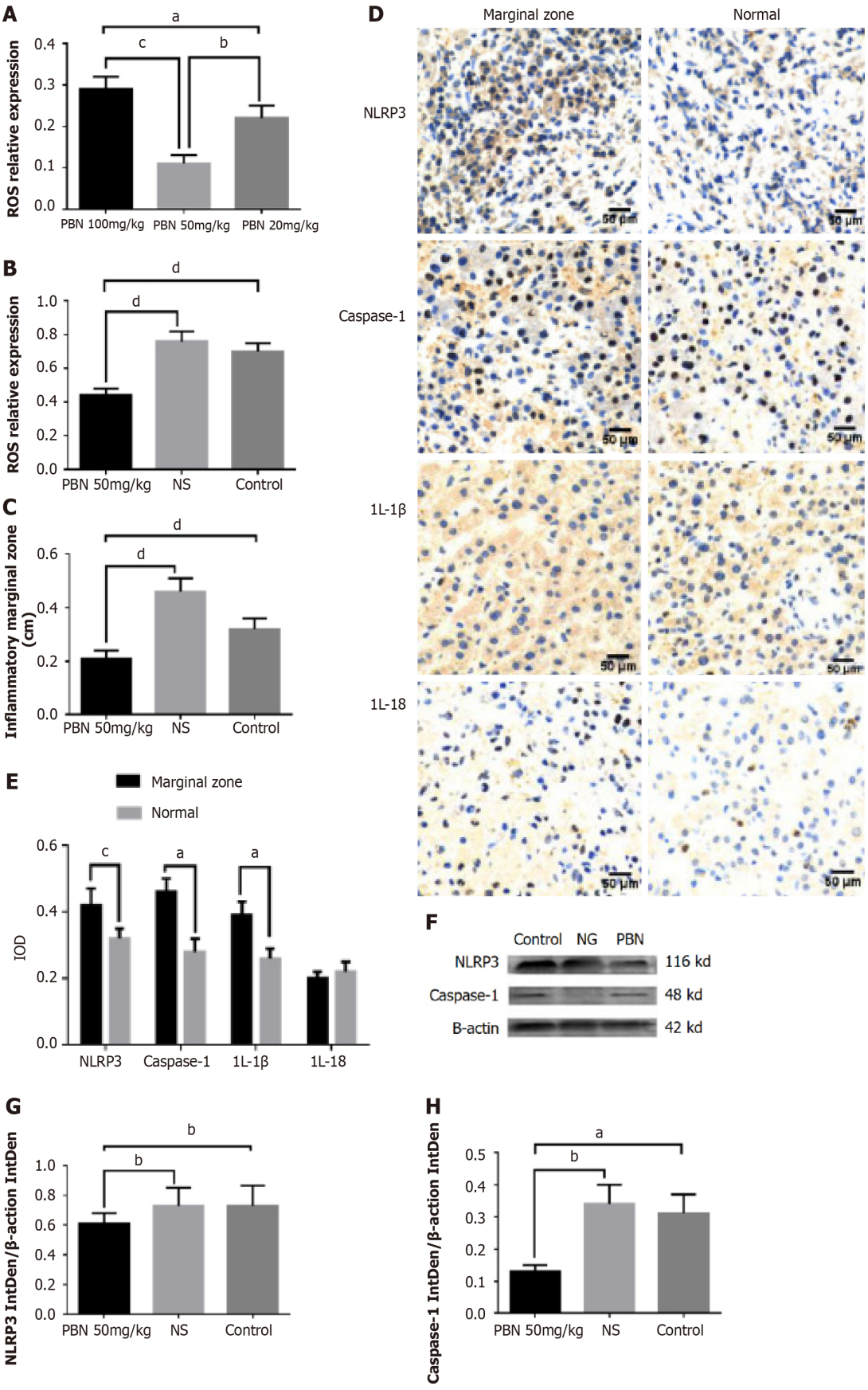


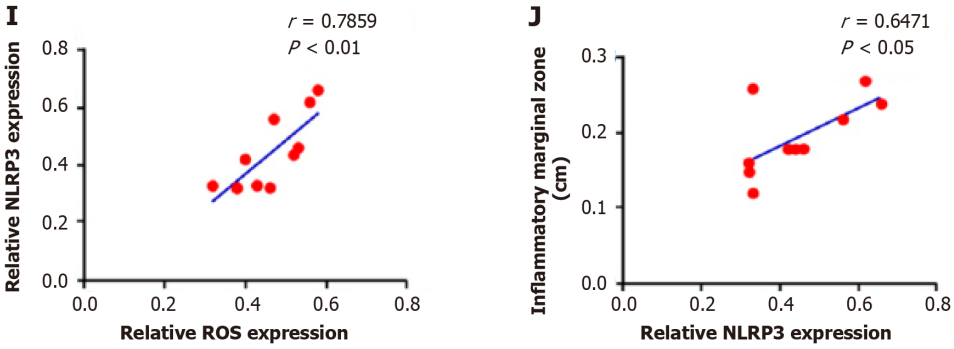
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Figure 2 Reactive oxygen species were highly produced in the hepatic alveolar echinococcosis marginal zone and were intimately associated with the activation of NOD-like receptor family pyrin domain-containing 3. A: Lesion growth in hepatic alveolar echinococcosis rats; B: Reactive oxygen species (ROS) production in the marginal zone and corresponding normal liver; C: Relative levels of ROS production; D: Relationship between ROS and NLRP3. C and D, n = 5 rats per group. $^{d}P < 0.0001$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; FITC-A: Fluorescein isothiocyanate isomer I-A.

response to infection and cellular damage. Multiple cellular events, including ionic flux, mitochondrial dysfunction, and ROS production, trigger its activation[48]. The aberrant activation of the NLRP3 inflammasome is closely related to various liver diseases, including liver cancer[45,46], viral hepatitis [49], non-alcoholic fatty liver disease[47,50], and parasitic diseases, such as schistosomiasis[51,52], leishmaniasis[53], malaria[54], and trypanosomiasis[55]. Our study confirms that the NLRP3 inflammasome plays a pivotal role in HAE *via* the NLRP3-caspase-1-IL-1β pathway involving inflammation in the marginal zone.

As important participants in oxidative stress, ROS are also closely related to inflammation[56]. The balance of ROS can regulate apoptosis and cell proliferation, activating a series of signal transduction pathways. Excessive ROS production damages cell integrity, resulting in tissue dysfunction[57]. ROS inhibitors significantly reduce this damage and alleviate acute liver injury. Consistent with a previous


A
ROS relative expression
PBN 100mg/kg
PBN 50mg/kg
PBN 20mg/kg
B
ROS relative expression
PBN 50mg/kg
NS
Control
C
Inflammatory marginal zone (cm)
PBN 50mg/kg
NS
Control
D
Marginal zone
Normal
NLRP3
Caspase-1
1L-1β
1L-18
50 μm
E
Marginal zone
Normal
IOD
NLRP3
Caspase-1
1L-1β
1L-18
F
Control
NG
PBN
NLRP3
116 kd
Caspase-1
48 kd
B-actin
42 kd
G
NLRP3 IntDen/β-action IntDen
PBN 50mg/kg
NS
Control
H
Caspase-1 IntDen/β-action IntDen
PBN 50mg/kg
NS
Control

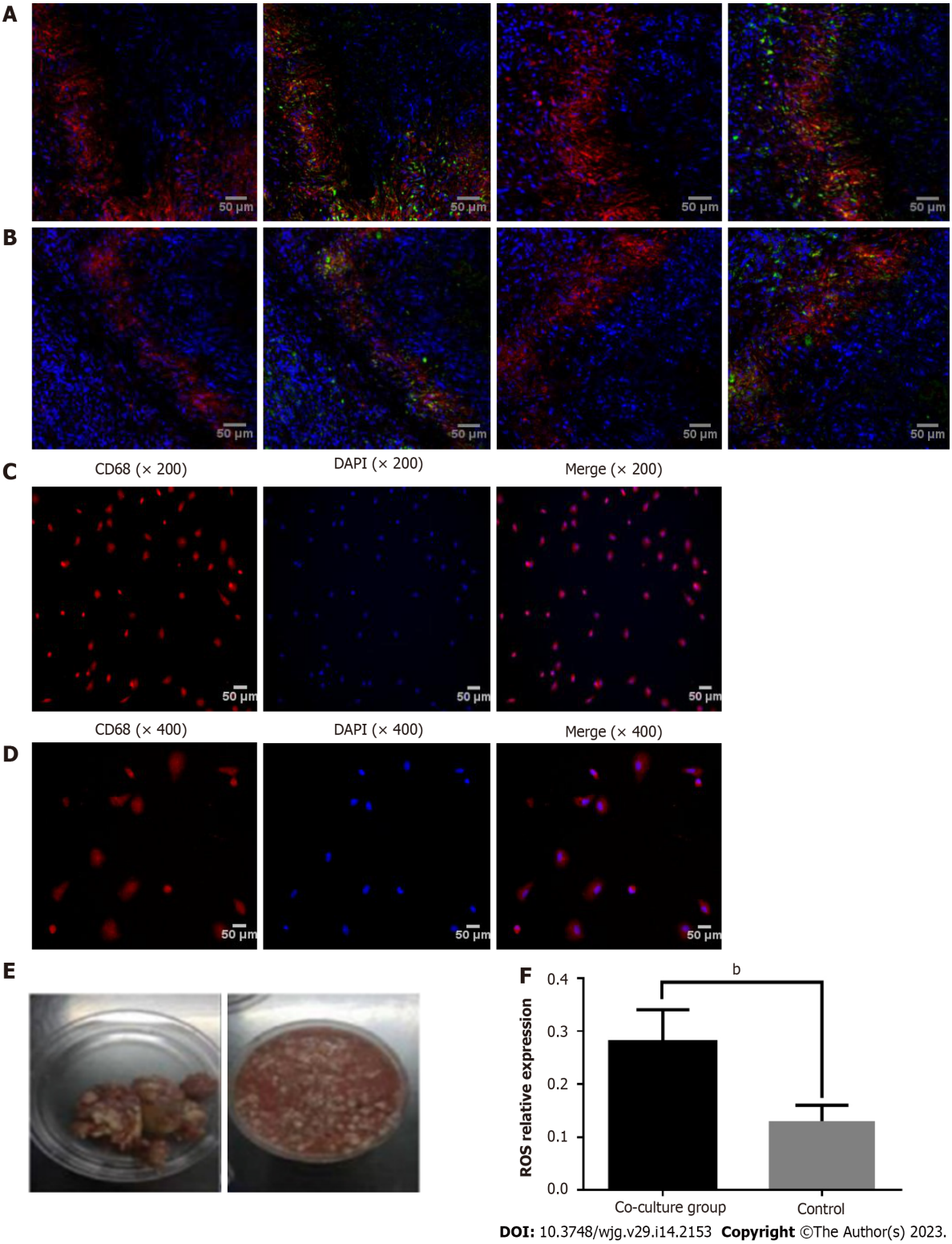


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Figure 3 Reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3 inflammasome activation in hepatic alveolar echinococcosis rats. A: Reactive oxygen species (ROS) production in the 20, 50, and 100 mg/kg/d N-tert-Butyl-α-phenylnitrone (PBN) groups; B: ROS production in the 50 mg/kg/d PBN, normal saline (NS), and control groups; C: Analysis of inflammation; D: Immunohistochemical staining of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18; E: Relative expression of NLRP3, caspase-1, IL-1β, and IL-18 in the marginal zone compared with the corresponding normal liver; F: Western blotting analysis of the PBN, NS, and control groups; G: NLRP3 IntDen/β-actin IntDen protein expression; H: Caspase-1 IntDen/β-actin IntDen protein expression; I: Relationship between ROS production and the relative expression of NLRP3; J: The relative expression of NLRP3 and inflammation observed in the marginal zone. Western blotting was performed in triplicate (mean ± SD). Scale bar, 50 μm. A, n = 3 rats per group; B, C, and E, n = 10 rats per group; I and J, n = 10. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; PBN: N-tert-Butyl-α-phenylnitrone; NS: Normal saline; IOD: Integral optical density; IL-1β: Interleukin-1β; IL-18: Interleukin-18.

study[45], our results showed that decreased ROS production in HAE inhibits NLRP3-caspase-1-IL-1β pathway activation and alleviates hepatocyte damage. ROS act as a critical regulator of various inflammatory processes and have received much attention[58]. In inflammatory liver disease, ROS induce fatty liver and ischemia/reperfusion injury by promoting inflammation and cell death[59]. In kidney disease, ROS is closely related to acute kidney injury in rats *via* ROS-mediated NLRP3 inflammasome activation [60]. In cardiovascular disease, ROS mediate several aspects of the stress-response signalling network [61]. In parasitic diseases, *Neospora caninum* evades immunity by inducing mitophagy and inhibiting pro-inflammatory cytokine production in a ROS-dependent manner[62]. Studies on parasitic diseases, inflammatory liver disease, cardiovascular disease, kidney disease, intestinal disease, and ischaemia/reperfusion injury also suggest that ROS production contributes to the activation of the NLRP3 inflammasome[63,64]. Although the specific mechanism of ROS-mediated NLRP3 inflammasome activation is unknown, the effect of ROS-mediated NLRP3 inflammasome activation under disease conditions has been confirmed[65]. Consistent with previous studies[61,64], our results indicated that NLRP3 inflammasome activation is intimately associated with ROS production. In addition, ROS-mediated NLRP3 inflammasome activation plays a vital role in the progression of inflammation in HAE. If the balance of ROS can be maintained by internal ROS scavengers and external antioxidants, ROS-mediated progression of inflammation and pathological processes can be alleviated or inhibited.

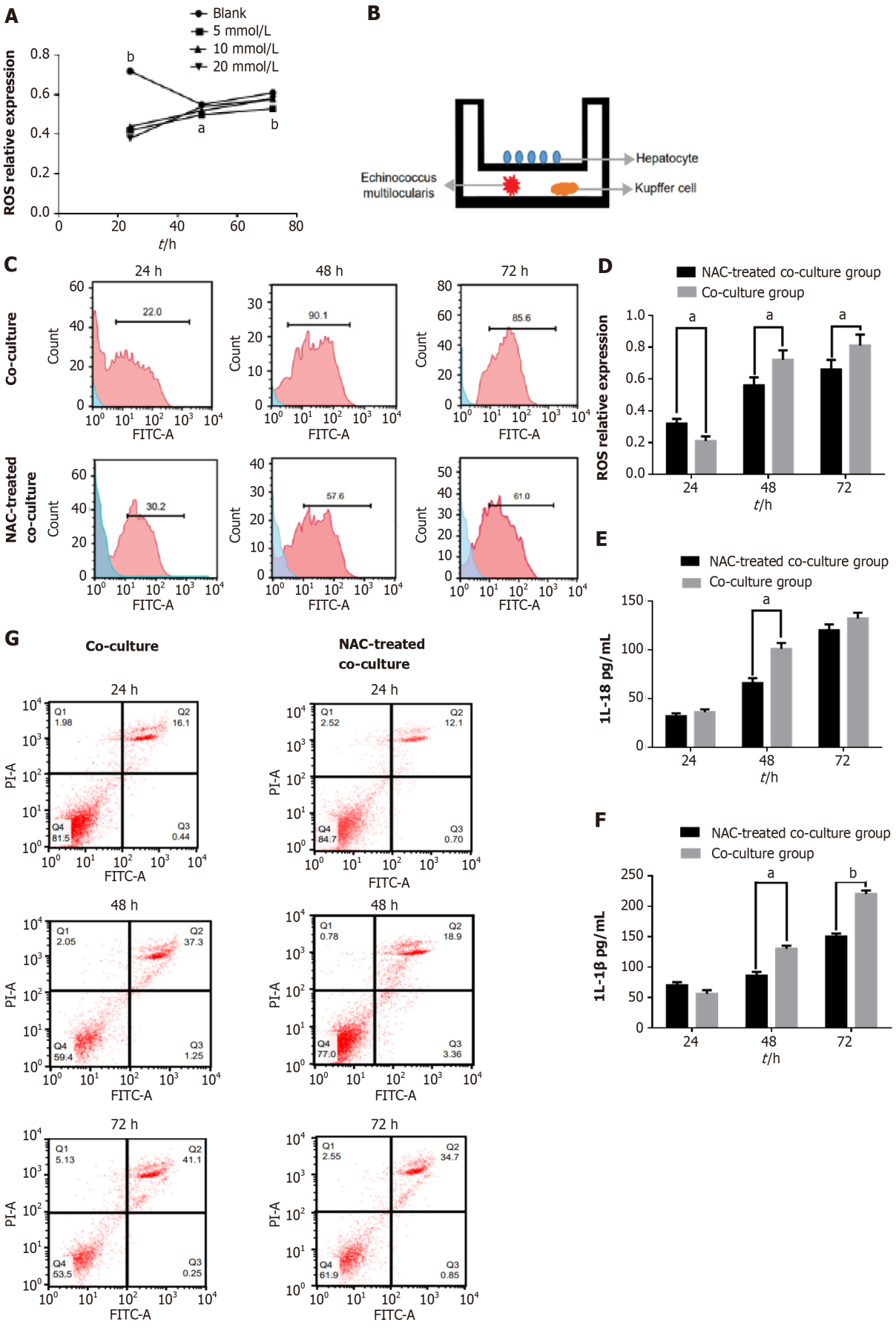
As NLRP3 inflammasome expression is transcriptionally regulated by NF-κB, it is very low in non-activated macrophages[11]. We localised cells expressing NLRP3 and caspase-1 in the marginal zone of HAE rats. The results showed that NLRP3 and caspase-1 were highly expressed in Kupffer cells (resident macrophages of the liver that play a leading role in the regulation of liver homeostasis). The marginal zone in HAE is a consequence of self-healing caused by continuous stimulation of *E. multilocularis*, and the inflammatory response is important in the progression of HAE. In the early stages of HAE, there are few abnormalities in liver function and liver enzyme levels. As the disease progresses, the imbalance of the internal environment of the liver and severe liver injury manifest in patients with mid-to-late-stage HAE as jaundice, abdominal pain, and even liver failure. Kupffer cells are associated with acute liver injury and secrete various cytokines under continuous stimulation by *E. multilocularis* [66]. IL-1β secreted by Kupffer cells is an important factor causing liver damage[64]. In a previous study [36], we observed typical chronic granulomatous changes around the lesion, accompanied by lymphocyte infiltration, hepatocyte degeneration and necrosis, and Kupffer cell proliferation and differentiation in tissues from patients with HAE. In this study, we used CD68 to identify Kupffer cells, and confirmed that they play an important role in the marginal zone. Kupffer cells are activated in response to liver injury. Activated Kupffer cells express markers of M1-like or M2-like macrophages, depending on external signals. Liver inflammation is regulated by the balance between pro-inflammatory M1 Kupffer cells and anti-inflammatory M2 Kupffer cells, which are partially self-renewal in a stable state [67]. We observed chronic granulomatous changes in the marginal zone of lesions in patients with HAE. Therefore, pro-inflammatory M1 Kupffer cells may be more mobile than anti-inflammatory M2 Kupffer cells in HAE. Activated Kupffer cells secrete IL-1β, a common pro-inflammatory cytokine, suggesting that pro-inflammatory M1 Kupffer cells play a key role in liver inflammation, although further research is needed.

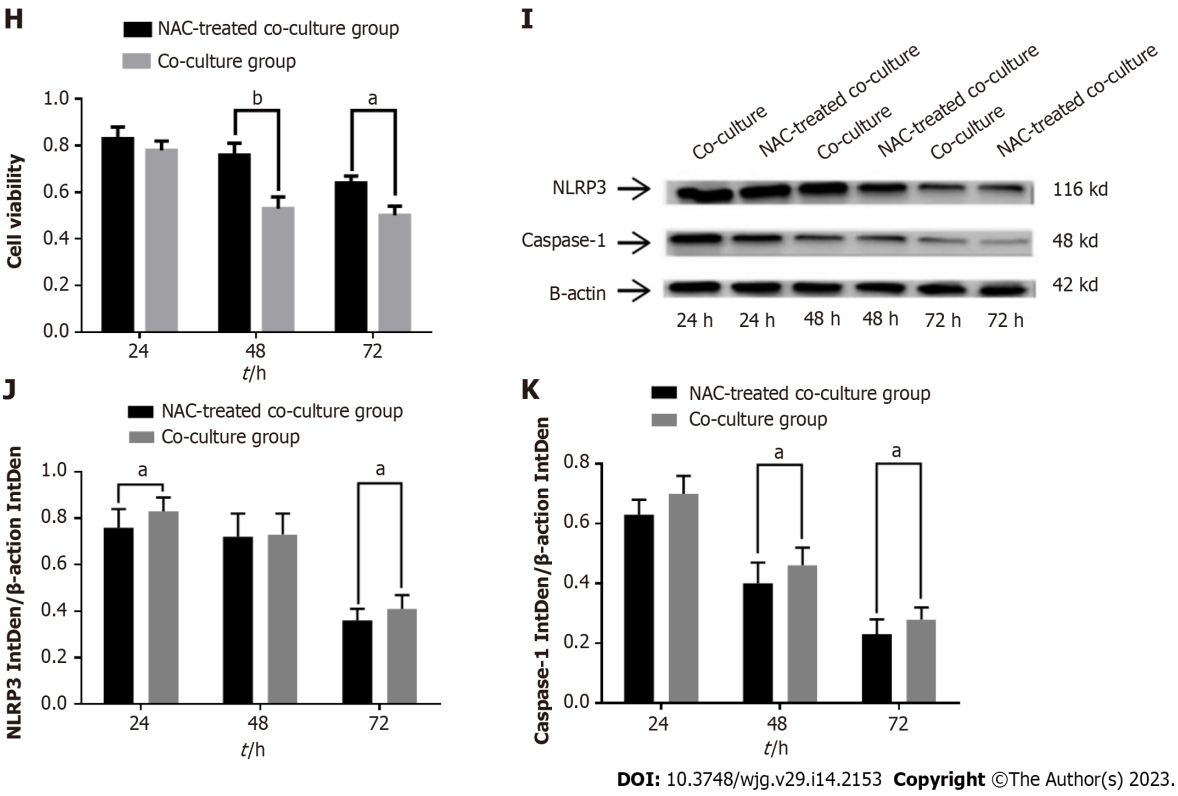


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Figure 4 *Echinococcus multilocularis* activation of the NOD-like receptor family pyrin domain-containing 3-caspase-1-interleukin-1β pathways in Kupffer cells. A: Cellular localisation of NOD-like receptor family pyrin domain-containing 3 (NLRP3) in the marginal zone (red denotes NLRP3 inflammasomes, blue denotes 4',6-diamidino-2-phenylindole (DAPI) stained nuclei, and green denotes the macrophage marker, CD68); B: Cellular localisation of caspase-1 in the marginal zone (red denotes caspase-1, blue denotes DAPI stained nuclei, and green denotes CD68); C: The identification of Kupffer cells at 200 × magnification and; D: The identification of Kupffer cells at 400× magnification (red denotes CD68 and blue denotes DAPI stained nuclei; the final image is a fusion image); E: Isolation of *E. multilocularis*; F: Relative expression in the co-culture and control groups. Scale bar, 50 μm. F, $n = 3$. $^{b}P < 0.01$. NLRP3: NOD-like receptor family pyrin domain-containing 3; DAPI: 4',6-diamidino-2-phenylindole.

Among the members of the inflammasome family, NLRP3 is the most well-characterised. The NLRP3 inflammasome is associated with several autoimmune and inflammatory diseases[68]. NLRP3 inflammasome activation leads to pro-inflammatory programmed cell death, known as pyroptosis[69]. NLRP3


A
Blank
5 mmol/L
10 mmol/L
20 mmol/L
ROS relative expression
t/h
B
Echinococcus multilocularis
Hepatocyte
Kupffer cell
C
24 h
48 h
72 h
Co-culture
NAC-treated co-culture
Count
FITC-A
22.0
90.1
85.6
30.2
57.6
61.0
D
NAC-treated co-culture group
Co-culture group
ROS relative expression
t/h
E
NAC-treated co-culture group
Co-culture group
1L-18 pg/mL
t/h
F
NAC-treated co-culture group
Co-culture group
1L-1β pg/mL
t/h
G
Co-culture
NAC-treated co-culture
24 h
48 h
72 h
PI-A
FITC-A
Q1 1.98
Q2 16.1
Q4 81.5
Q3 0.44
Q1 2.52
Q2 12.1
Q4 84.7
Q3 0.70
Q1 2.05
Q2 37.3
Q4 59.4
Q3 1.25
Q1 0.78
Q2 18.9
Q4 77.0
Q3 3.36
Q1 5.13
Q2 41.1
Q4 53.5
Q3 0.25
Q1 2.55
Q2 34.7
Q4 61.9
Q3 0.85



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Figure 5 Reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3-caspase-1-interleukin-18 pathway activation in Kupffer cells. A: Reactive oxygen species (ROS) production with the indicated N-acetyl-L-cysteine (NAC) dose at the specified time points. $^{b}P <$ 0.01, blank *vs* 5 mmol/L; $^{a}P < 0.05$, 10 mmol/L *vs* 5 mmol/L. $^{b}P < 0.0120$ mmol/L *vs* 5 mmol/L; B: Representation of the Transwell model used in this study; C: ROS production in the co-culture groups treated with or without NAC at the indicated time points; D: Relative production of ROS; E: Interleukin (IL)-18 expression; F: IL-1β expression; G: Apoptosis of hepatocytes in the co-culture groups treated with or without NAC at 24, 48, and 72 h; H: Cell viability; I: Western blotting analysis of the indicated proteins; J: NLRP3 IntDen/β-actin IntDen protein expression; K: Caspase-1 IntDen/β-actin IntDen protein expression. $^{a}P < 0.05$; $^{b}P < 0.01$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; FITC-A: Fluorescein isothiocyanate isomer I-A; PI: Propidium Iodide; NAC: N-acetyl-L-cysteine; IL-1β: Interleukin-1β; IL-18: Interleukin-18.

inflammasome activation results in caspase-1 activation, which triggers the release of pro-inflammatory cytokines, IL-1β and IL-18. NLRP3 inflammasome activation has received widespread attention, and significant progress has been made in understanding the molecular mechanisms underlying the priming step of NLRP3 inflammasome activation. NLRP3 inflammasome activation is triggered by several cellular signals: Mitochondrial dysfunction, ROS production, potassium and calcium ion signalling, and lysosomal rupture[48]. As HAE is a parasitic disease that mainly affects the liver, we considered the modes of NLRP3 inflammasome activation in liver and parasitic diseases. In non-alcoholic fatty liver disease, free fatty acids induce ROS production, which has been proposed as a common mechanism of NLRP3 inflammasome activation, resulting in hepatocyte injury and steatosis[68,69]. In viral hepatitis, NLRP3 inflammasome activation is mediated by elevated ROS production, resulting in liver inflammation and hepatocellular pyroptosis under hydrogen peroxide stress[70]. Wei *et al*[46] showed that 17β-estradiol-induced NLRP3 inflammasome activation inhibited liver cancer by triggering apoptosis and inhibiting protective autophagy. Additionally, Ma *et al*[71] showed that NLRP3 inflammasomes play an important role in host defence against *Talaromyces marneffei* (*T. marneffei*) infection *via* the Dectin-1/Syk signalling pathway, *T. marneffei* yeast triggers NLRP3-ASC-caspase-1 inflammasome assembly to facilitate IL-1β maturation. While NLRP3 inflammasome activation plays different roles at various stages of leishmaniasis, ROS-mediated NLRP3 inflammasome activation has been confirmed[32]. Schistosomiasis attracted our attention as a parasitic disease that mainly affects the liver. In a study of *Schistosoma japonicum* (*S. japonicum*), Zhang *et al*[72] showed that *S. japonicum* induces liver fibrosis *via* NF-κB signaling and NLRP3 inflammasome activation in Kupffer cells, promoting cytokine production and the mechanism of NLRP3 inflammasome activation during *S. japonicum* infection was found to be dependent on ROS production and lysosomal activity. NLRP3 expression is closely related to an increase in ROS production and the degree of inflammation in the marginal zone. Therefore, we hypothesised that *E. multilocularis* activates the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, promoting IL-1β synthesis and release, leading to hepatocyte damage. We showed that *E. multilocularis* alone triggers ROS production in Kupffer cells, and reduces the impact of ROS production in a co-culture system, effectively inhibiting IL-1β production and reducing the apoptosis of rat hepatocytes.

IL-1β, mainly synthesised by macrophages, is an important pro-inflammatory cytokine that activates lymphocytes, macrophages, and natural killer cells, and is involved in various pathological processes [73]. IL-1β is activated in neurological diseases (especially Parkinson's disease and Alzheimer's disease), intestinal diseases, and cancer[74,75]. Previous studies have led to the development of IL-1β-targeted therapies, which have achieved considerable success[76]. IL-1β is also associated with parasitic infections[20,21]. IL-1β may eliminate parasites by associating with other components of the immune system[77]. IL-1β can also co-ordinate innate and adaptive immune responses to eliminate pathogens. However, excess IL-1β leads to inflammatory diseases[78]. As mentioned above, IL-1β expression may be critical in inflammatory diseases, and blocking IL-1β may be a useful strategy for treating inflammatory diseases. We confirmed that IL-1β levels were increased in HAE and may be responsible for the development of HAE infection. We did not observe a corresponding increase in IL-18 Levels, possibly because the ROS-mediated NLRP3-caspase-1-IL-18 pathway was not activated in HAE.

Patients do not show obvious clinical symptoms in the early stages of HAE. Patients begin treatment in the mid-to-late stages of the disease, which are often accompanied by jaundice, abdominal pain, abnormal liver function, and declining immune system function. Radical surgery is the first choice for the treatment of alveolar echinococcosis. There is no doubt that choosing the correct treatment is key to improving the outcome of HAE. From previous studies[79,80], we have accumulated experience in the surgical treatment of HAE, and patients with blood vessel invasion (hepatic vein, portal vein, or inferior abdominal vein) and lymph node metastasis were still given the opportunity to undergo surgery. Similar to liver cancer lesions, HAE lesions are usually characterised by milky white surfaces. However, the specimens are harder and denser than cancer. Most lesions have clear boundaries. Some small lesions are distributed around the large lesions. Liquefaction necrosis is observed in the center of the larger lesions, with a distinct marginal zone around the lesion. However, unlike patients with liver cancer who often benefit from immunotherapeutic drugs, few drugs are available for the treatment of HAE. Drugs that protect the liver, maintain stable liver function, and inhibit parasites (*e.g.*, albendazole) are routine choices, owing to the lack of evidence from pathophysiological research on the occurrence and development of HAE. An obvious pathological change in the development of HAE is inflammation. We begin with the NLRP3 inflammasome, an important factor in the inflammatory signaling pathway. We explored the mode of activation and the effect of downstream products on HAE. ROS-mediated NLRP3 inflammasome activation was a key factor leading to hepatocyte injury and triggering a cascade of inflammatory reactions. There are a number of challenges in translating findings into the clinic. However, the use of dietary antioxidants to inhibit oxidative stress and reduce ROS production may be a possible treatment option. Therefore, ROS production may be a promising target for the treatment of HAE.

Herein, the expression of NLRP3, caspase-1, and IL-1β was significantly elevated in the marginal zone in patients with HAE. To the best of our knowledge, this is the first study to explore the effect and mechanism of ROS-mediated NLRP3 inflammasome activation in HAE. *In vivo* experiments showed that inhibiting ROS production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation in the HAE marginal zone. *In vitro* data revealed significantly decreased apoptosis rates in hepatocytes, corresponding to reduced ROS-mediated NLRP3-caspase-1-IL-1β pathway activation in Kupffer cells. However, our study has a few limitations. We did not verify whether M1 Kupffer cells or M2 Kupffer cells were more mobile in the regulation of inflammatory response, nor did we further explore the activation modes of NLRP3 inflammasome. Additionally, although NLRP3 inflammasome is the most well-characterised inflammasome, further studies are warranted to determine whether other inflammasomes are activated in the HAE marginal zone. We conclude that *E. multilocularis* induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells. The increase in ROS production caused by *E. multilocularis* may be a potential mechanism of NLRP3 inflammasome activation. In addition, crosstalk between signaling pathways may be involved in the regulatory network in the marginal zone. Future studies should clarify the function and mechanisms of the immune responses in the progression of HAE. This will enhance our understanding of the occurrence and development of infection and facilitate the development of diagnostics and therapy.

CONCLUSION

Our *in vivo* experiments showed that inhibiting ROS production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation in the HAE marginal zone. *In vitro* data revealed significantly decreased apoptosis rates in hepatocytes, corresponding to reduced ROS-mediated NLRP3-caspase-1-IL-1β pathway activation in Kupffer cells. We conclude that *E. multilocularis* induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells.

ARTICLE HIGHLIGHTS

Research background

In recent years, the role of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in parasitic diseases has attracted widespread attention. However, the role and clinical significance of the NLRP3 inflammasome in Hepatic alveolar echinococcosis (HAE) remain unclear.

Research motivation

To investigate the mechanism of NLRP3 inflammasome activation in HAE and the effect of its downstream products may enhance our understanding of the occurrence and development of infection.

Research objectives

To investigate the NLRP3 inflammasome and its mechanism of activation in HAE.

Research methods

We assessed the expression of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE. A rat model of HAE was employed to investigate the role of the NLRP3 inflammasome in the marginal zone of HAE. Transwell experiments were conducted to investigate the effect of *Echinococcus multilocularis* (*E. multilocularis*) in stimulating Kupffer cells and hepatocytes. Furthermore, immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay were used to evaluate NLRP3, caspase-1, IL-1β, and IL-18 expression; flow cytometry was used to detect apoptosis and reactive oxygen species (ROS).

Research results

NLRP3 inflammasome activation was significantly associated with ROS. Inhibition of ROS production decreased NLRP3-caspase-1-IL-1β pathway activation and mitigated hepatocyte damage and inflammation.

Research conclusion

E. multilocularis induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, indicating that ROS may serve as a potential target for the treatment of HAE.

Research perspectives

Although NLRP3 inflammasome is the most well-characterised inflammasome, further studies are warranted to determine whether other inflammasomes are activated in the HAE marginal zone and clarify the function and mechanisms of the immune responses in the progression of HAE.

FOOTNOTES

Author contributions: Chen CS and Fan HN conceived and designed the experiments; Chen CS, Zhang YG, and Wang HJ collected the samples; Chen CS and Zhang YG performed the experiments and data analyses; Chen CS wrote the first draft of the manuscript; Zhang YG and Fan HN provided comments for the revisions; all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Changes in the gut mycobiome in pediatric patients in relation to the clinical activity of Crohn's disease

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Abstract

BACKGROUND

Numerous studies have shown that in Crohn's disease (CD), the gut microbiota is of great importance in the induction and maintenance of inflammation in the gastrointestinal tract. Until recently, studies have focused almost exclusively on bacteria in the gut. Lately, more attention has been paid to the role of intestinal fungi.

AIM

To study the gut mycobiome analysis of pediatric patients with CD (in different stages of disease activity) compared to healthy children.

METHODS

Fecal samples were collected from patients: With active, newly diagnosed CD (n = 50); active but previously diagnosed and treated CD (n = 16); non-active CD and who were in clinical remission (n = 39) and from healthy volunteers (n = 40).

Fungal DNA was isolated from the samples. Next, next generation sequencing (MiSeq, Illumina) was performed. The composition of mycobiota was correlated with clinical and blood parameters.

RESULTS

Candida spp. were overrepresented in CD patients, while in the control group, the most abundant genus was *Saccharomyces*. In CD patients, the percentage of *Malassezia* was almost twice that of the control ($P < 0.05$). In active CD patients, we documented a higher abundance of *Debaryomyces hansenii* (*D. hansenii*) compared to the non-active CD and control ($P < 0.05$) groups. Moreover, statistically significant changes in the abundance of *Mycosphaerella*, *Rhodotorula*, and *Microidium* were observed. The analyses at the species level and linear discriminant analysis showed that in each group it was possible to distinguish a specific species characteristic of a given patient population. Moreover, we have documented statistically significant correlations between: *D. hansenii* and patient age (negative); *C. zeylanoides* and patient age (positive); *C. dubliniensis* and calprotectin (positive); *C. sake* and calprotectin (positive); and *C. tropicalis* and pediatric CD activity index (PCDAI) (positive).

CONCLUSION

Mycobiome changes in CD patients, and the positive correlation of some species with calprotectin or PCDAI, give strong evidence that fungi may be of key importance in the development of CD.

Key Words: Intestinal mycobiome; Fungi; Crohn's disease; Inflammatory bowel disease; Next generation sequencing; Molecular microbiology

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Core Tip: There is growing evidence that intestinal microorganisms are associated with pathogenesis of Crohn's disease (CD). Until recently, studies have focused almost exclusively on bacteria. In this study we showed alterations in the fungal composition in pediatric patients with CD. Changes within the specific species of fungi depending on disease activity, and the positive correlation of some species with calprotectin or pediatric CD activity index, give strong evidence that these microorganisms may be of key importance in the development and course of CD. Some fungal species can be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD.

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INTRODUCTION

Crohn's disease (CD) belongs to the group of chronic gastrointestinal tract disorders known as inflammatory bowel diseases (IBD), which are characterized by a relapsing and remitting course. The pathogenesis of CD is not fully understood, but it is supposed that its development and severity are influenced by a combination of genetic, immune and microbiological disorders[1,2]. Intestinal microorganisms are increasingly indicated as one of the key factors in the etiology of IBD[1,3-5].

The human gut is colonized by a stable and abundant community of microorganisms, collectively referred to as the gut microbiota, consisting of bacteria, archaea, fungi, protozoans, and viruses[6]. These microbes have physiological functions associated with nutrition, regulation of immune homeostasis, and protection of the host against pathogenic bacteria[3,6]. Recent advances in molecular techniques have recognized alterations in the composition, abundance, and function of the microbiota in CD, which is known as dysbiosis[1,6]. To date, it has not been established whether the changes in the microbiota are a direct cause of the disease or only a secondary effect of the chronic inflammatory process and the treatment applied. However, numerous studies in animal and human models[7-10] have shown that the gut microbiota in CD is of great importance in the induction and maintenance of inflammation in the gastrointestinal tract.

Until recently, studies have focused almost exclusively on bacteria in the gut[4,11-13]. Lately, more attention has been paid to the role of intestinal fungi. These microorganisms, their metabolites, and their interaction with bacterial populations may directly or indirectly influence the host's immune response

[1]. Fungi interact with the immune system among others *via* Dectin-1, which is one of the most important pattern recognition receptors[7,14]. This receptor has an influence on the activation of macrophages, neutrophils, and dendritic cells. Dectin-1 recognizes β-1.3 glucans that are components of the fungal cell wall and thus activates the intracellular signal that leads to the production of inflammatory cytokines[15]. It has been suggested that Dectin-1 polymorphisms are strongly associated with an excessive immune response to commensal fungi and thus may contribute to the development of CD [7]. In addition, the induction of anti-*Saccharomyces cerevisiae* antibodies (ASCA) that recognize yeast cell wall mannans is a serological biomarker frequently present in patients with CD, which may suggest that the disease may be associated with an unusual interaction of the immune system with fungi.

Taking into account the likely relationship between the mycobiota and CD, the aim of this study was a detailed taxonomic analysis of the fungal composition in children with CD. In our study, we analyzed the differences between the mycobiomes of CD patients and healthy children, as well as the changes that occur in patients' mycobiomes depending on disease activity.

MATERIALS AND METHODS

Study population

Pediatric patients with CD, aged 2 to 18 years, hospitalized at the University Children's Hospital in Krakow in the years 2016–2019, were recruited for the study. Diagnosis was made on the basis of the clinical picture, as well as endoscopic, histopathological, and radiological examinations, according to the revised Porto criteria[16]. The study protocol was approved by the Jagiellonian University Bioethics Committee (No. 1072.6120.21.2020). Disease phenotype was evaluated according to the Paris criteria [17]. The patients were recruited into two research groups on the basis of clinical disease activity according to the pediatric CD activity index (PCDAI).

Patients with active CD, PCDAI > 10 points.

In this group, two subgroups were distinguished: Ia–patients with newly diagnosed CD (before the implementation of any treatment); and Ib–patients previously diagnosed and treated with aminosalicylates (5-ASAs) and/or azathioprine.

Patients with non-active CD, PCDAI ≤ 10 points.

This group (II) included patients who were previously diagnosed with CD and treated with 5-ASAs and/or azathioprine and were in clinical remission at the time of inclusion in the study.

The exclusion criteria were: Lack of consent to participate in the study; patients under 2 or over 18 years of age; use of antibiotics and/or probiotics and/or antifungal drugs within 30 d before collecting stool samples; confirmed infections of the gastrointestinal tract; isolated perianal fistula.

The control group included healthy non-hospitalized children and adolescents without antibiotic, antifungal, or probiotic treatment during the 30-d period before stool sample collection.

Samples

At inclusion, routine laboratory tests evaluating biochemical parameters and calprotectin were performed and the PCDAI score was evaluated in all patients. Stool samples from all participants were obtained at the University Children's Hospital in Krakow and immediately frozen at -80 °C, then delivered under deep freeze conditions to the Department of Microbiology of Jagiellonian University Medical College in Krakow, where DNA was isolated and further analyses were performed.

Isolation of DNA from stool samples

The stool samples were thawed and 150 mg of each material was used for fungal DNA isolation using the Genomic Mini AX Stool Kit (A&A Biotechnology, Gdansk, Poland), according to our previously described modification[18,19].

The isolates were measured with a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, United States) in the A260 nm and A260 nm/280 nm ratios to determine the purity and concentration of DNA.

Preparation of genomic library

The DNA extracted was used to perform polymerase chain reaction (PCR) amplification (T100 Thermal Cycler, BioRad, California, United States), with primers targeting the ITS-1 regions of the fungal rDNA gene[20] to prepare ITS libraries.

The primer sequences were the following: [Adapter sequences for MiSeq sequencer (Illumina, San Diego, United States) are written in bold][21]:

Forward primer:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAAAAGTCGTAACAAGGTTTC;

Reverse primer:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTCAAAGAyTCGATGATTCAC.

The composition of the reaction mixture was as follows: 5 μL of DNA, 12.5 μL of Kapa Biosystems (Roche, Basel, Switzerland), 0.5 μL of each primer (20 mmol/L, Genomed, Warsaw, Poland), 6.5 μL of water (A&A Biotechnology, Gdansk, Poland). Thermal cycling conditions included an initial denaturation at 95 °C for 5 min followed by 50 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

The volume of 5 μL of each amplicon was subjected to electrophoretic separation on 1.5% agarose gel (Prona, Basica Le, Burgos, Spain) diluted 10 times with TBE buffer (Sigma-Aldrich, Saint Louis, United States). The PCR products were visualized in the FastGene FAS–Digi Pro (Nippon Genetics, Duren, Germany) in the presence of UV light, to check library quality. Subsequent steps (purification, sample indexing, sample quantification, and pooling) were prepared according to the Illumina library preparation protocols[21].

Next generation sequencing

The library concentration was measured using PicoGreen fluorescent dye (Thermo Fisher, Waltham, MA, United States) and pooled with 30% spike-in PhiX control DNA (Illumina, San Diego, United States). Next, the pooled libraries were applied to the Reagent Kit V3 cartridge (600 cycles) (Illumina, San Diego, United States) and the sequencing was carried out using MiSeq (Illumina, San Diego, United States) at the Department of Clinical Immunology of the University Children's Hospital in Krakow.

Negative control (containing water instead of DNA) and positive control (MSA 1010 Mycobiome Genomic DNA mix, ATCC, Manassas, United States) were included throughout the genomic library procedure and next generation sequencing sequencing.

Bioinformatics analysis

The ITS-targeted amplicon pair reads were demultiplexed on the basis of the unique molecular barcodes. The short read of raw data was collected as FASTQ files. Then, the per-sample raw taxonomic classification was performed using the Illumina 16S Metagenomics workflow, on the basis of the UNITE Fungal ITS Database v7.2.

Sequences were clustered into operational taxonomic units (OTUs) at a similarity cutoff value of 97%-98%. This classification was performed on the basis of the algorithm described by Wang *et al*[20]. Next, the metagenomic reads were aligned to the UNITE Fungal ITS Database v7.2 reference taxonomy database. During this step, the candidate fungal reads were identified, and the sequences which did not match the reference fungal ITS database were filtered out (these were features with counts below 4 counts, 20% of prevalence filter). In the normalization step, sequences were trimmed for low-quality scores (less than 3). Finally, filtered and normalized reads were matched again to the fungi, and a definitive taxonomic assignment was performed. Abundance profiling was characterized on the basis of the OTU data, which were compared at different taxonomic levels based on the annotations. The diversity within samples was measured on the basis of alpha and beta diversity indices. To calculate the diversity within samples (richness and evenness), Observed, ACE, Chao1, Shannon, Simpson, and Fisher matrices were applied, and analysis of variance (ANOVA) statistics were performed. Beta diversity analysis was performed on the basis of Principal Coordinate Analysis using Bray–Curtis and the Jaccard index. The statistical significance of sample grouping was tested using the Permutational MANOVA (PERMANOVA) statistical method. The linear discriminant analysis (LDA) Effect Size (LEfSe) algorithm was used to discover statistically significant biomarkers. The Kruskal–Wallis rank sum test was performed to describe features with significant abundance at different taxonomic levels. A 0.05 *P* value cutoff and 2.0 LDA score cutoff were established to determine the most significant features.

RESULTS

Characteristics of the study population

One hundred and five pediatric patients were included in the study. The control group consisted of 40 children. The characteristics of the study groups are presented in Table 1.

DNA quality

A DNA purity of ≥ 1.7 was assumed. All samples examined met this criterion.

Analysis of the biodiversity of the gut mycobiota

The data obtained by sequencing consisted of 4859784 reads (1680 minimum reads per sample, 79543 maximum) with an average number of reads of 33748. All samples were included in the analysis.

Alpha diversity at the phylum level (L2) was different and statistically significant between: Ia *vs* control (expressed as the Shannon, Chao, and Fisher indices; Ia *vs* II (Fisher index); and II *vs* control (Chao index) (Table 2). Moreover, we observed statistically significant differences in alpha diversity at the species level (L7) between groups: Ia *vs* II (expressed as both Chao and Fisher indices); Ia *vs* control (Chao and Fisher indices); Ib *vs* II (Fisher index); Ib *vs* control (Fisher index) (Table 2).

Table 1 Clinical data from patients with Crohn's disease and healthy subjects

	Group Ia (*n* = 50)	Group Ib (*n* = 16)	Group II (*n* = 39)	Control (*n* = 40)
General characteristics				
Male:Female (ratio)	29:21 (1.38)	8:8 (1)	22:17 (1.29)	15:25 (0.6)
Age (years), mean ± SD	13 ± 2.9	15 ± 2.8	12.5 ± 4.3	11 ± 4.1
BMI (kg/m^2), mean ± SD	17.0 ± 3.1	18.1 ± 2.7	18.1 ± 3.2	18.3 ± 3.8
PCDAI, mean ± SD	34.4 ± 12.7	38.2 ± 18.6	4.1 ± 3.9	N/A
Disease distribution by Paris classification (number of patients)				
A1a	4	2	7	
A1b	46	14	30	
A2	0	2	2	
L1	2	1	2	
L2	6	2	7	
L3	16	4	12	
L4aL1	8	2	6	
L4aL2	8	3	5	
L4aL3	10	4	7	
B1	39	11	27	
B2	11	5	12	

Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects; Paris classification: Age: A1a (0–10 yr), A1b (10–17 yr), A2 (17–40 yr); Location: L1 (distal 1/3 ileum and/or limited cecal disease), L2 (colonic), L3 (ileocolonic), L4a (upper disease proximal to ligament of Treitz), L4b (upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum); Behaviour: B1 (non-structuring non-penetrating), B2 (structuring), B3 (penetrating), B2B3 (both penetrating and structuring disease either at the same or different times); BMI: Body mass index; PCDAI: Pediatric Crohn's disease activity index; N/A: Not applicable.

Table 2 Alpha diversity metrics *P* values at the phylum (L2) and species (L7) level, expressed as Shannon, Chao and Fisher indices

Index/group	Shannon		Chao		Fisher	
	L2 level	L7 level	L2 level	L7 level	L2 level	L7 level
Ia *vs* Ib	0.713	0.251	0.836	0.121	0.533	0.312
Ia *vs* II	0.606	0.584	0.228	0.001[a]	0.000[a]	0.011[a]
Ia *vs* control	0.000[a]	0.313	0.006[a]	0.000[a]	0.000[a]	0.000[a]
Ib *vs* II	0.492	0.128	0.356	0.185	0.132	0.041[a]
Ib *vs* control	0.072	0.053	0.103	0.077	0.072	0.027[a]
II *vs* control	0.086	0.710	0.044[a]	0.631	0.998	0.938

[a]$P < 0.05$, statistically significant value.
Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects.

Furthermore, statistically significant differences were observed at the phylum and species level for beta diversity between groups Ia *vs* control for three indices. Additionally, differences at the species level were observed between groups II and control (expressed as the Jensen–Shannon index) (Table 3).

Analysis of the composition of the gut mycobiota

The gut mycobiota was evaluated at six taxonomic levels (phylum, class, order, family, genus, species). Three of these, L2 (phylum), L6 (genus), and L7 (species) were described in this study to include the most representative taxa. At the phylum level (L2), all OTUs obtained were assigned to two main phyla:

Table 3 Beta diversity metrics *P* values at the phylum (L2) and species (L7) level, expressed as Bray-Curtis, Jensen-Shanon and Jaccard indices

Index/group	Bray-Curtis		Jensen-Shannon		Jaccard	
	L2 level	L7 level	L2 level	L7 level	L2 level	L7 level
Ia *vs* Ib	0.753	0.727	0.746	0.92	0.813	0.632
Ia *vs* II	0.409	0.291	0.404	0.176	0.469	0.389
Ia *vs* control	0.038[a]	0.006[a]	0.009[a]	0.001[a]	0.035[a]	0.008[a]
Ib *vs* II	0.532	0.374	0.543	0.468	0.517	0.332
Ib *vs* control	0.172	0.086	0.125	0.064	0.15	0.094
II *vs* control	0.313	0.057	0.206	0.026[a]	0.393	0.087

[a]$P < 0.05$, statistically significant value.

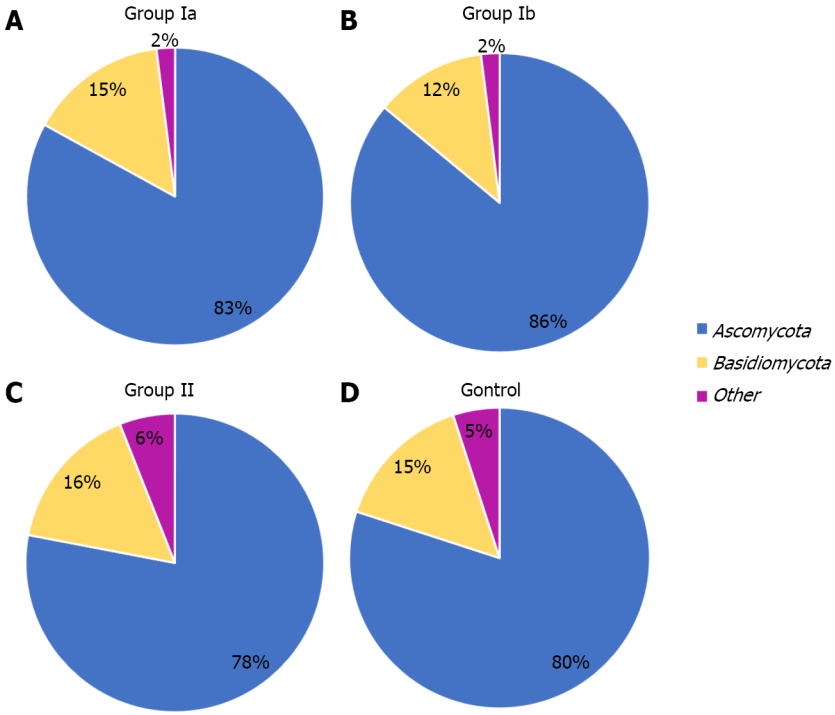
Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects.

Ascomycota and *Basidiomycota*. The others were non-fungal and belonged mainly to plant taxa. The *Ascomycota* phylum was clearly predominant in all groups, respectively: 83% in group Ia; 86% in group Ib; 78% in group II; 80% in the control group (Figure 1). In groups Ia and Ib, the percentage of these fungi was significantly higher compared to the control group ($P = 0.039$; $P = 0.046$, respectively).

At the genus level (L6) (Figure 2), *Candida* dominated in all groups including CD patients (Ia–46%, Ib–45%, II–44%), and in groups Ia and II the abundance was statistically higher compared to the control group (26%; respectively: $P < 0.001$; $P = 0.035$). On the other hand, in the control group, the dominant genus was *Saccharomyces* (38%), and the abundance of this fungus was significantly higher in comparison to Ia (27%; $P < 0.001$). Interestingly, in all groups that included CD patients, the prevalence of *Malassezia* was almost twice as high (11%–14%) compared to the control group (7%), although statistically significant differences were only documented between group Ia and control (14% *vs* 7%, $P = 0.015$). Furthermore, in the group of patients with active CD (Ia and Ib), *Debaryomyces* constituted a higher percentage (respectively: 5%; 6%) compared to the other groups (2%), but significant differences were noted only between group Ia and the control ($P = 0.024$). Additionally, a significantly higher level of *Rhodotorula* was observed in the control group (9%) compared to the groups Ia (1%, $P < 0.001$), Ib (0.3%, $P = 0.001$), and II (2%, $P = 0.039$), and in group group Ib compared to group II (0.3% *vs* 2%, $P = 0.014$). Moreover, a significantly lower level of *Mycosphaerella* was observed in group Ib (0.3%) compared to groups II (5%, $P = 0.015$) and control (1%, $P = 0.016$). Statistical differences were also observed between group II (5%) and control (1%, $P = 0.027$) and for group Ia and II (2% *vs* 5%, $P = 0.029$). Statistical differences for *Microidium* were also found between groups Ib and control (2% *vs* 4%, $P < 0.001$).

At the species level (L7), *S. cerevisiae* and *Candida albicans* (*C. albicans*) were dominant in all investigated groups and quantitatively constituted nearly 50% of all fungi. In the control group, *S. cerevisiae* was the most abundant yeast (38%), in contrast to the group of patients with CD (23% in Ia; 28% in Ib; 20% in II), in whom an increased percentage of individual *Candida* species was observed (Figure 3). In groups Ia, II, and control, the dominant species of the genus *Candida* was *C. albicans* (respectively 23%; 32%; 17%), and within this species, statistically significant differences were observed between groups Ia *vs* Ib (23% *vs* 11%, $P = 0.008$) and Ib *vs* control (11% *vs* 17%, $P = 0.008$). Differences close to statistical significance were documented between group II and the control (32% *vs* 17%, $P = 0.054$). The mycobiome of the patients in group Ib was overrepresented by *Candida dubliniensis* (19%) and *Candida zeylanoides* (12%) and the percentage of these fungi was significantly higher than in group II (respectively: 6%, $P = 0.001$; 1%, $P = 0.003$). A higher percentage of *Candida tropicalis* (10%) was observed in group Ia compared to the other groups (0.1%–4%), although statistically significant changes were found between groups Ia and Ib (10% *vs* 0.1%, $P = 0.008$) and Ib and control (0.1% *vs* 2%, $P = 0.021$). Furthermore, among the control group, a statistically higher percentage of *Candida krusei* (*C. krusei*) was observed compared to group II (2% *vs* 1%, $P = 0.005$).

Patients with CD (especially those newly diagnosed with active disease) were characterized by a higher prevalence of *Malassezia restricta* compared to the control group (11% *vs* 6%, Figure 3); however, these changes were not statistically significant. Statistical differences were observed between groups Ib and II (10% *vs* 9%, $P = 0.001$). Among controls, a significantly higher percentage (9%) of *Rhodotorula mucilaginosa* (*R. mucilaginosa*) was found in comparison to Ia (0.7%, $P = 0.001$); Ib (0.3%, $P = 0.002$) and II (2%, $P = 0.027$). Statistically significant differences were also found for groups Ib and II ($P = 0.015$). In the control group, a slight increase in *Saccharomyces cariocanus* was observed in comparison to group II (3% *vs* 2%, $P = 0.046$) and for *Microidium phyllanthi* (*M. phyllanthi*) compared to all CD groups (4% *vs* 2%, $P < 0.05$). There were also statistically significant differences in *Mycosphaerella tassiana* (*M. tassiana*)



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Figure 1 Fungal profiles at the phylum level (L2). A: Active/newly-diagnosed Crohn's disease (CD); B: Active/previously diagnosed and treated CD; C: Non-active CD; D: Healthy subjects.

abundance between groups Ia *vs* II (2% *vs* 5%, $P = 0.029$) and Ib *vs* control (0.3% *vs* 0.6%, $P = 0.016$). In addition, the changes in abundance of *Debaryomyces hansenii* (*D. hansenii*) (3% *vs* 1%, $P = 0.020$) and *Malassezia globosa* (*M. globosa*) (2% *vs* 5%, $P = 0.037$) were documented between groups Ia and II, and in *M. globosa* between groups II *vs* control (5% *vs* 1%, $P = 0.030$), and also in *Epicoccum nigrum* (*E. nigrum*) between Ia *vs* control (2% *vs* 0.7%, $P = 0.015$).

Linear discriminant analysis-determination of biomarkers

Owing to LDA we have selected species of fungi that could be biomarkers characterizing and distinguishing a given group of patients (Figure 4). We have shown that *C. tropicalis* was overrepresented in group Ia compared to the other groups ($P = 0.026$). Whereas increased richness of *C. albicans* ($P = 0.005$), *M. globosa* ($P = 0.011$) and *M. tassiana* ($P = 0.024$) was documented in group II compared to the other groups. The fungal biomarkers that differentiated the control group from the CD patient groups were *R. mucilaginosa* ($P < 0.001$), *C. krusei* ($P = 0.013$), and *M. phyllanthi* ($P < 0.001$). Group Ib was characterized by a higher abundance of *E. nigrum* compared to the other groups ($P = 0.011$).

Correlation between the abundance of fungi and patients' clinical parameters

The analysis of the abundance of fungal species showed significant correlations between: *D. hansenii* and *C. zeylanoides* and patient age (negative and positive, respectively); *C. dubliniensis* and *C. sake* and calprotectin (both positive) and a positive correlation between *C. tropicalis* and PCDAI (Table 4).

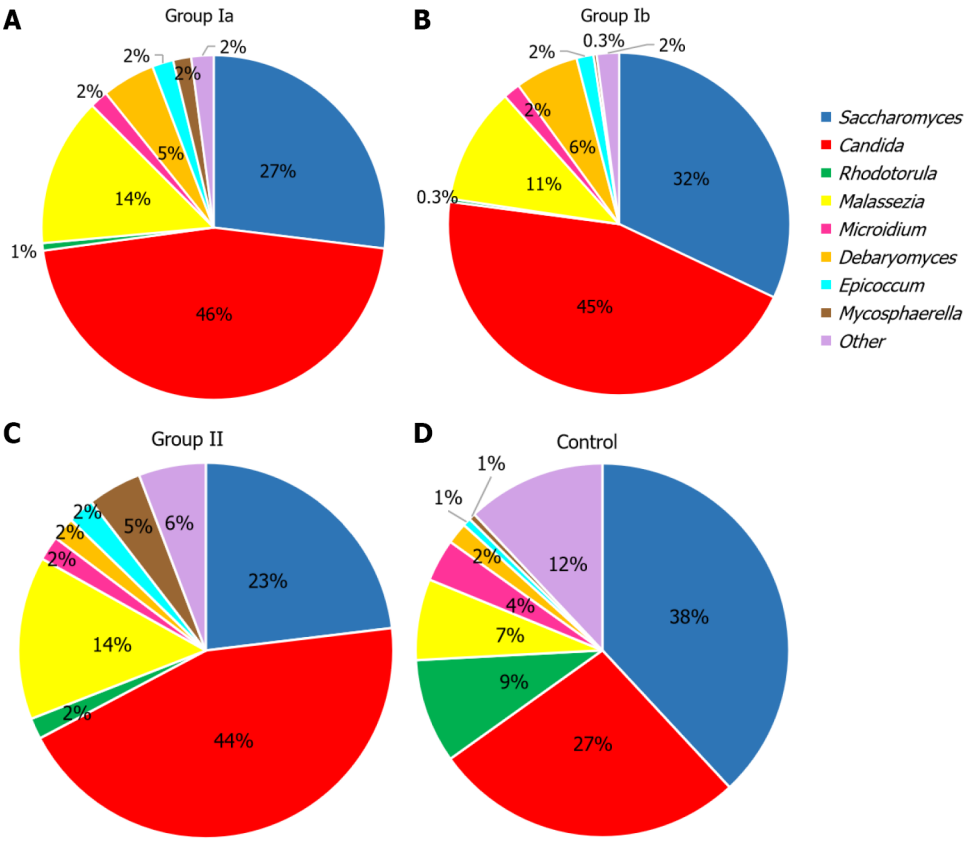
DISCUSSION

Numerous studies suggest that changes in the intestinal microbiota and the interaction between microorganisms and the host may lead to the development of IBD. The main limitation of the available studies[12,22-24] is the predominance of experiments in adult patients who have been treated for a long time and who have been sick for many years, which could significantly affect the results obtained. Moreover, the studies of the intestinal microbiota in IBD conducted so far have focused primarily on disorders within the bacteriobiome. There is a lack of more comprehensive research, including fungi, which constitute a very important component of the intestinal microbiome[25]. In our study, we recruited children and adolescents with both newly and previously diagnosed CD and we compared their mycobiome in the exacerbation and remission periods. In addition, we recruited healthy children into a control group. Such a study allowed a more reliable determination of the relationship between fungi in the digestive tract and the course of CD. Moreover, the main exclusion criterion in all groups

Table 4 Correlations of fungal species with patient parameters

Variables/age		Variables/calprotectin		Variables/PCDAI	
Debaryomyces hansenii	$P < 0.008$	*Candida dubliniensis*	$P < 0.003$	*Candida tropicalis*	$P < 0.006$
	$r = -0.08$		$r = 0.29$		$r = 0.58$
Candida zeylanoides	$P < 0.004$	*Candida sake*	$P < 0.04$		
	$r = 0.08$		$r = 0.19$		

PCDAI: Pediatric Crohn's disease activity index.



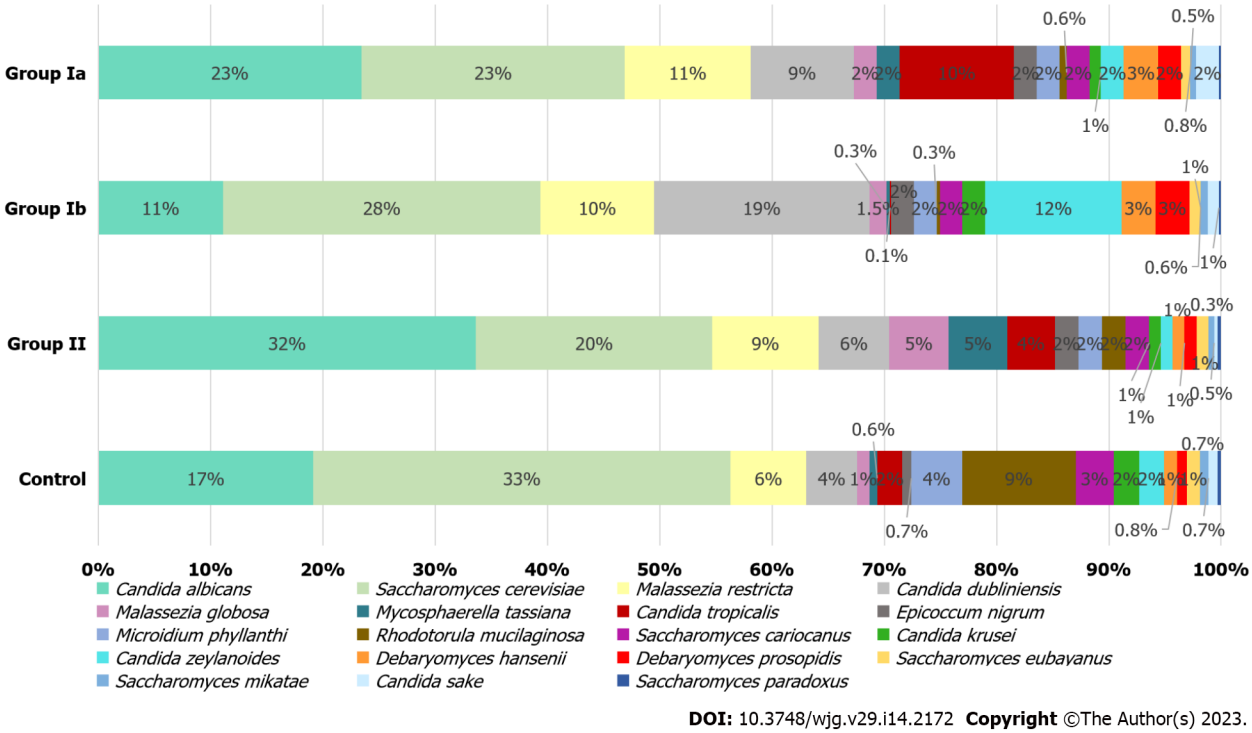
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Figure 2 Fungal profiles at the genus level (L6). A: Active/newly-diagnosed Crohn's disease (CD); B: Active/previously diagnosed and treated CD; C: Non-active CD; D: Healthy subjects.

was the use of antibiotics, probiotics, and antifungal drugs that could significantly change the composition of the mycobiome. Thus, the impact of confounding factors associated with antimicrobial therapy was eliminated. Our data have shown that the mycobiota of pediatric CD patients was different in comparison to healthy controls, and also depended on the stage of disease activity.

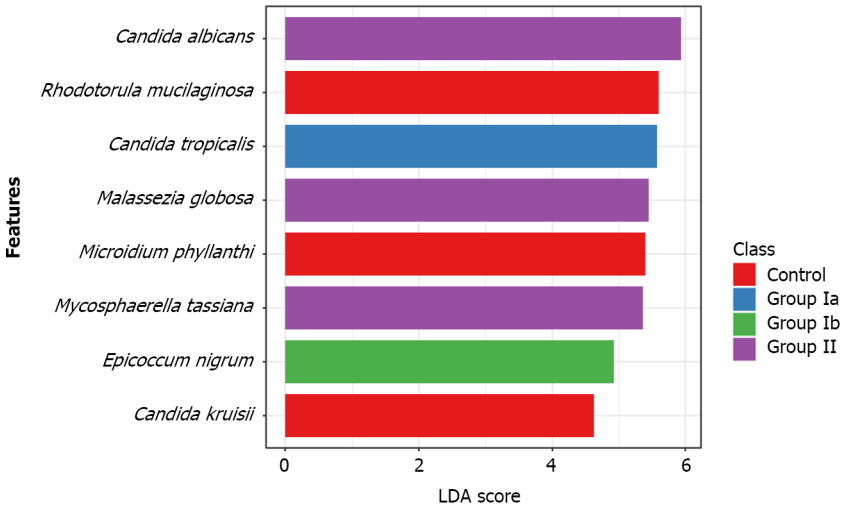
The alpha diversity (which is a measure of biodiversity within the study groups) of the fungal microbiota was significantly different (both at the phylum and species levels) in group Ia compared to the control expressed as the Chao and Fisher indices (Table 2). Additionally, biodiversity was higher in control compared to the Ia group for the three analyzed indices. Lower alpha diversity in the samples from patients with active, newly diagnosed CD indicated a reduction in fungal biodiversity, *i.e.*, lower number of taxons in a single sample. These data are consistent with previous reports[24,26,27]. Interestingly, we have shown that patients in the non-active phase of CD (II) were characterized by significantly higher alpha biodiversity indices at the level L7 (species) compared to patients with newly diagnosed, active disease (Ia). This provides the basis for the conclusion that while patients achieve remission, normalization of mycobiome occurs, an indicator of which is the increase in the measure of biodiversity[28].

The analysis of beta diversity (which is a measure of differences between the study groups) has shown that there were statistically significant differences between group Ia and the control, both at the



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Figure 3 Fungal profiles at the species level (L7). Group Ia: Active/newly-diagnosed Crohn's disease (CD); Group Ib: Previously diagnosed and treated CD; Group II: Non-active CD; Control: Healthy subjects.



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Figure 4 Histogram of the linear discriminant analysis logarithmic scores for statistically different fungi between study and control groups. Positive linear discriminant analysis scores represent fungi that were over-abundant in Group Ia–active/newly diagnosed Crohn's disease (CD) (blue bars), Group Ib–active/previously diagnosed and treated CD (green bars), Group II–non-active CD (purple bars), and control–healthy subjects (red bars) groups.

L2 and L7 levels (expressed by the three indices) (Table 3). These results indicate a significantly different taxonomic composition at the phylum and species levels between these groups. The different composition of mycobiota seen in the patients of group Ia compared to healthy children indicates that dysbiosis appears at the very beginning of the disease. However, the treatment (groups Ib and II) likely reduces the differences in mycobiome composition compared to controls.

At the phylum level, *Ascomycota* was predominant in all groups (Figure 1), but in patients with active CD (irrespective of whether it was a newly diagnosed or previously diagnosed disease), the percentage of this phylum was significantly higher (83%–86%) compared to the control group (80%). These observations are consistent with studies by other researchers showing an increased percentage of *Ascomycota* in adult patients with active CD[22,29].

At the genus level (Figure 2), we observed that among patients with CD (irrespective of disease activity), *Candida* was the predominant genus in contrast to the control group (significant differences between Ia *vs* control and II *vs* control; $P < 0.05$). Analyses at the species level (Figure 3), showed that in each of the studied groups it was possible to distinguish a specific species within the genus *Candida*, characteristic of a given patient population. Thus, an excessive abundance of *C. tropicalis* has been observed in newly diagnosed patients in active phase of CD (group Ia) (Figure 3). Interestingly, in patients in remission (group II), the abundance of this species decreased more than double. Moreover, in the control group, *C. tropicalis* was 5 times lower than in group Ia. This may suggest that *C. tropicalis* may be a biomarker of inflammation in the gut in the course of CD, which was supported by LDA analysis (Figure 4). It has previously been shown that overrepresentation of *C. tropicalis* is commonly found among patients with active CD[12,23,30] and possibly influences the initiation and maintenance of inflammation[23]. Hoarau *et al*[23] showed that a higher prevalence of *C. tropicalis* fungi was positively correlated with levels of ASCA and with the abundance of *Serratia marcescens* and *Escherichia coli*. Researchers demonstrated that these microorganisms interact with each other in the gut, forming a biofilm that can promote an excessive immune response and cause intestinal mucosal barrier dysfunction, contributing to the formation of inflammatory lesions[23]. Additionally, studies in animal models of IBD confirmed that *C. tropicalis* has strong immunogenic potential. Supplementation in mice with *C. tropicalis* resulted in excessive secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha, interferon gamma and, interleukin-17, and then intensification of inflammation in the gut [7]. In addition, in other independent studies, *C. tropicalis* has induced dysbiosis that involved changes in the presence of mucin-degrading bacteria, leading to altered tight junction protein expression with increased intestinal permeability. Then, it induced a strong Th1/Th17 response, leading to an accelerated pro-inflammatory phenotype in experimental colitis mice[31]. Interestingly, in our research, a positive correlation between *C. tropicalis* and PCDAI was observed, which strengthens the above suggestions that these fungi may be associated with the exacerbation of the disease.

Analysis at the species level has shown that *C. albicans* constituted the highest percentage in the group of patients in remission (II) compared to the other groups (Figure 3), and LDA analysis has documented that this species may be a fungal biomarker for patients in non-active CD ($P = 0.005$, Figure 4). Previous studies showed that these yeasts were more abundant in CD patients, and it was suggested that *C. albicans* may promote IBD by increasing the inflammatory response[27,32,33]. However, it should be noted that these studies did not take into account patients in remission. Patients in the active phase or all CD patients together (undifferentiated according to the phase of disease activity) were compared with healthy people. In our research, an additional comparison of the exacerbation and remission groups has provided evidence that there are more *C. albicans* in non-active CD compared to active disease. It is likely that the treatment used to lead the patient into remission somehow promotes the growth of *C. albicans*; however, the effect of anti-inflammatory treatment commonly used in CD (like 5-ASAs; azathioprine) on the mycobiome has not been proven so far. Moreover, the growth of fungi in the gut is closely related to changes in the bacterial microbiota, so it is also possible that changes in the bacterial community that occur during remission contribute to the increased abundance of *C. albicans*[12,23,34-36].

The results of our study show an increased number of *C. dubliniensis* and *C. zeylanoides* in active but already treated patients (Ib) compared to other groups (Figure 3). To our knowledge, this is the first such report. Perhaps the increase in the colonization by these species of fungi was related to the treatment or a long-term inflammatory process. However, it should be noted that among treated patients in remission (II), the abundance of *C. dubliniensis* and *C. zeylanoides* significantly decreased ($P = 0.001$, $P = 0.003$; respectively) compared to treated patients in the active phase (Ib). It is possible that colonization by these fungi may depend on the disease activity and long-term inflammatory process that promotes their multiplication. Although *C. dubliniensis* is not an epidemiologically significant species, some reports indicate that it may cause opportunistic infections that may be associated with a higher degree of intestinal colonization[37,38]. Thus, its involvement in the maintenance of inflammation in CD cannot be ruled out, especially since a positive significant correlation of this species with calprotectin (which is a marker of intestinal inflammation) was observed (Table 4).

C. zeylanoides are fungi found in food products[39-41], so their presence may be associated with different diets of patients. On the other hand, we documented that these fungi were positively significantly correlated with age (Table 4). Perhaps, the higher abundance of *C. zeylanoides* in group Ib was related to the fact that we had slightly elderly patients in this group compared to the other one (Table 1). There are several reported cases in which *C. zeylanoides* caused fungemia[42-44]; however, there is no evidence that this microorganism has a connection with chronic or autoimmune diseases.

An interesting subject of study is *D. hansenii*. The abundance of these fungi was higher among patients with active CD, however, statistically significant changes were only observed between groups Ia and II ($P = 0.020$). The fact that the prevalence of this species was three times higher in patients with active CD compared to patients in remission and control (Figure 3) indicates that this fungus may be involved in maintaining intestinal inflammation or even inducing disease. Jain *et al*[45] came to similar conclusions. The researchers showed that damaged intestinal tissue (in a mouse model of CD) was over-colonized with *D. hansenii*, which consequently impeded the healing and regeneration process of intestinal crypts. Interestingly, when mice received an antifungal drug (amphotericin), the wounds

began to heal[45]. In the same study, the authors conducted an analysis with the participation of adult people with CD and showed that *D. hansenii* was detected in all samples taken from patients (100%), but only in one healthy person (10%). Further detailed observation of tissue biopsies showed that this fungus was present only in the inflamed mucosa, whereas it was absent from the non-inflamed mucosa sampled from the same patient[45]. Our study is the first report documenting the presence of *D. hansenii* in children and adolescents with CD. Furthermore, our data indicate that the abundance of these fungi depends on the phase of disease activity. These observations lead to the conclusion that *D. hansenii* may be involved in maintaining inflammation.

In this study, we have documented that CD patients were over-colonized with *Malassezia* species compared to the control group (Figures 2 and 3). This is consistent with the observations of other researchers[12,27,46]. Patients with CD often suffer from malabsorption syndrome, manifested by fatty diarrhea. *Malassezia* requires lipids to multiply; therefore, it is possible that increased detectability of these yeasts in CD patients is related to the fact that excess fat in the stool creates an optimal environment for *Malassezia* over-colonization. However, the participation of these yeasts in maintaining inflammation cannot be excluded because there are a few studies that demonstrate the immunogenic properties of *Malassezia* in the context of IBD[46,47]. The species analysis provided interesting observations regarding the changes in individual species of *Malassezia* depending on the activity of the disease (Figure 3). Thus, among children in remission (II), *M. globosa* colonization increased and these changes were statistically significant compared to group Ia ($P = 0.037$) and the control group ($P = 0.030$). On the other hand, patients in the exacerbation phase had a higher percentage of *M. restricta* ($P = 0.001$). The participation of *M. restricta* was previously reported in individual studies. Limon *et al*[46] demonstrated that these fungi were significantly more abundant in CD patients than in healthy subjects, and in addition, their presence was associated with exacerbation in a mouse model of IBD[46]. Furthermore, the team showed that these yeasts were especially present in patients with IBD caspase recruitment domain-containing protein 9 (*CARD9*) risk. CARD9 is a signaling adapter protein that is essential for antifungal innate immunity in mice and humans. Single nucleotide polymorphism in the gene for CARD9 is the strongest genetic risk factor linked to IBD. A certain variant of this gene caused human immune cells to produce more potent inflammatory cytokines in response to *M. restricta*[46]. Additionally, Li *et al*[12] showed that the biopsies collected from inflamed mucosa were over-colonized by *M. restricta* in relation to tissue without inflammation from the same patient[12]. A study on the immunogenicity of fungi of the genus *Malassezia* in the context of IBD confirmed that both *M. globosa* and *M. restricta* have strong pro-inflammatory properties. Both yeast species induced the production of inflammatory cytokines by dendritic cells in large part mediated through Dectin2 and CARD9 signaling [47]. Furthermore, *M. restricta* exacerbated colitis in germ-free mouse models of IBD triggered by inflammatory reactions mediated by CARD9[46]. The above data indicate that fungi of the genus *Malassezia* significantly influence the host's immune response, so their role in the pathogenesis of IBD cannot be ruled out. Understanding the species changes that occur in the various phases of disease activity, which were documented in our analysis, forms the basis for further research and gives an insight into the role of these yeasts in the course of CD.

Another species whose participation in CD is worth considering, is *Epicoccum nigrum*. These fungi have been overlooked so far, but this is probably due to the difficulties in culturing and detecting these yeasts. In this study, we have shown that the abundance of these fungi was significantly higher in CD patients (Ia) compared to healthy children ($P = 0.015$, Figure 3). Wheeler *et al*[48] documented the first reports on *E. nigrum* in CD and suggested its association with the treatment applied. The researchers showed that extended pretreatment with antifungal drugs restructured the intestinal fungal population. Increased colonization by the *E. nigrum* fungi, among others, has been observed. It is unknown whether the observed changes were due to alterations in the microbial community that promoted *E. nigrum* overgrowth, or whether these yeasts were resistant to the applied treatment. However, these changes were correlated with exacerbated disease in chemically induced models of experimental colitis[48]. In our study, the exclusion criterion was the use of antifungal drugs, so the presence of *E. nigrum* was not related to treatment. However, it cannot be ruled out that the alterations observed in the microbial community in CD patients promote the overgrowth of *E. nigrum*. Fungal populations of the intestinal tract directly or indirectly help to maintain healthy intestinal homeostasis. Any changes in the composition of the mycobiome may cause serious disruption, especially in genetically susceptible people to diseases (*e.g.* predisposed to CD). On the other hand, the LDA analysis (Figure 4) has shown that *E. nigrum* may be a fungal biomarker for patients with active, previously diagnosed disease (Ib). Perhaps the presence of these fungi is associated with the active long-term inflammation. It cannot be ruled out that chronic and long-term inflammatory lesions in the intestine favor the growth of this species. The overrepresentation of *E. nigrum* found in this report in CD patients requires further research.

In this study, we also demonstrated an increase in colonization level by *R. mucilaginosa* and *M. phyllanthi* among healthy subjects compared to CD patients ($P < 0.05$, Figure 3). LDA analysis has shown that these species may be a fungal biomarker for healthy children (Figure 4). *R. mucilaginosa* are fungi commonly found in soil, plants, and animals; however, they are also food contaminants. They are often isolated from fruit juices and dairy products[49]. The increase in the occurrence of these fungi in healthy children could be related to a varied and rich diet used by healthy people. In contrast, patients with CD

usually are on a dairy-free diet and exclude many products from their meals, so they carry fewer yeasts associated with foods in the digestive tract. On the other hand, *M. phyllanthi* are plant pathogens that can be delivered to the gastrointestinal tract through the consumption of vegetables and fruits. Patients with CD significantly reduce their consumption of legumes, and stone fruits, and thus, they have a lower abundance of plant-related fungi than healthy people following a varied diet[50,51].

CONCLUSION

In conclusion, this study confirms alterations in intestinal fungal composition in pediatric patients with CD and shows that some species of fungi may be a microbiological marker related to the activity of the disease. In patients with CD, we have documented an increased load of fungi with potential pro-inflammatory effects (*e.g.*, *Candida* spp., *Malassezia* spp.)[7,14,23,46], while fungi with potential anti-inflammatory effects (such as *Saccharomyces*)[23,52-55] were found in a lower percentage. Moreover, changes within the specific species of fungi depending on disease activity, and the positive correlation of some species with calprotectin or PCDAI, give strong evidence that these microorganisms may be of key importance in the development and course of CD.

Furthermore, we have shown that some fungal species can be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD. Further research should focus on selecting fungal species that could be biomarkers to help predict disease exacerbation. Moreover, future research should assess whether the mycobiota could be a therapeutic target.

ARTICLE HIGHLIGHTS

Research background

The recurrent and chronic course of Crohn's disease (CD), its systemic after effects, and intestinal complications constitute a serious clinical problem, and since the pathogenesis of the disease is unknown, causal treatment is currently not used. As CD incidence age keeps falling and there is a growing number of cases, we are led to undertake intensive studies to determine the possible causes of this disease. Recently, due to the growing interest in the topic of intestinal microbiome, a hypothesis has emerged that the initiation of CD is associated with dysbiosis within the gut microbiota. And while the importance of bacteria in the pathogenesis has been, up to now, a common subject of research, the involvement of fungi has usually been overlooked. The few available studies including mycobiome analysis concern adults and not children, previously treated patients, or those with long-term disease. These shortcomings distort the results due to the impact of confounding variables (such as treatment, age, or the long-term disease process) on changes in the fungal composition.

Research motivation

Undoubtedly, the composition of the microbiome has a significant influence on maintaining internal balance and health and microbial changes constitute an important factor inducing pathological processes. Due to the fact that fungi are an important component of the gut microbiota, it is possible that alterations in the composition of the gut mycobiome may have an impact on the induction of CD.

Research objectives

Taking into account the likely relationship between the mycobiota and the host, the aim of this study was to perform a detailed taxonomic analysis of the fungal composition in pediatric patients with CD. In our study, we recruited children and adolescents with newly and previously diagnosed CD and compared their mycobiome in the exacerbation and remission periods. Additionally, we recruited healthy children into a control group. Such a study allowed for a more reliable determination of the relationship between fungi in the digestive tract and the course of CD.

Research methods

DNA was isolated from stool samples from patients: With active, newly diagnosed CD (n = 50); active but previously diagnosed and treated CD (n = 16); non-active CD who were in clinical remission (n = 39) and healthy volunteers (n = 40). The next step was to prepare genomic libraries for next generation sequencing (NGS). NGS was performed using a MiSeq sequencer (Illumina). The composition of the gut mycobiota was analyzed using UNITE Fungal ITS Database v7.2, and then correlated with clinical and blood parameters.

Research results

Our study confirms alterations in fungal composition in pediatric CD patients and shows that some species of fungi may be a kind of microbiological marker related to the activity of the disease. In CD

patients, we have documented an increased load of fungi with potential pro-inflammatory effects (*e.g.* *Candida* spp., *Malassezia* spp.), while fungi with potential anti-inflammatory effects (such as *Saccharomyces*) were found in a lower percentage. Interestingly, the greatest alterations in mycobiome composition (compared to the control group) were observed among newly diagnosed patients, before implementing any therapeutic approaches. This is strong evidence that fungi may play an important role in the development of CD. This thesis is supported by the fact that a positive correlation of some species with calprotectin or pediatric CD activity index was documented. Furthermore, owing to linear discriminant analysis, we have shown that some fungal species could be biomarkers characterizing and distinguishing a given group of patients (depending on the disease activity) which in the future may be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD.

Research conclusions

Changes in the composition of the intestinal mycobiome occur already at the beginning of the disease (in newly diagnosed and untreated patients). Furthermore, the composition of fungi changes depending on the activity of CD.

Research perspectives

Further research should focus on selecting fungal species that could be biomarkers to help predict disease exacerbation. Furthermore, next research should assess whether the fungal mycobiota could be a therapeutic target.

FOOTNOTES

Author contributions: Krawczyk A performed the molecular investigations, interpreted the data, prepared the tables and figures, and wrote the manuscript; Kowalska-Duplaga K recruited patients, and revised the paper; Zapała B analyzed data, and revised the paper; Książek T performed the investigations; Drażniuk-Warchoł M recruited patients; Salamon D, and Gosiewski T coordinated the study, interpreted data, and revised the article; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Acoustic radiation force impulse predicts long-term outcomes in a large-scale cohort: High liver cancer, low comorbidity in hepatitis B virus

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Abstract

BACKGROUND

Acoustic radiation force impulse (ARFI) is used to measure liver fibrosis and predict outcomes. The performance of elastography in assessment of fibrosis is poorer in hepatitis B virus (HBV) than in other etiologies of chronic liver disease.

AIM

To evaluate the performance of ARFI in long-term outcome prediction among different etiologies of chronic liver disease.

METHODS

Consecutive patients who received an ARFI study between 2011 and 2018 were enrolled. After excluding dual infection, alcoholism, autoimmune hepatitis, and others with incomplete data, this retrospective cohort were divided into hepatitis B (HBV, n = 1064), hepatitis C (HCV, n = 507), and non-HBV, non-HCV (NBNC, n = 391) groups. The indexed cases were linked to cancer registration (1987-2020) and national mortality databases. The differences in morbidity and mortality among the groups were analyzed.

RESULTS

At the enrollment, the HBV group showed more males (77.5%), a higher prevalence of pre-diagnosed hepatocellular carcinoma (HCC), and a lower prevalence of comorbidities than the other groups ($P < 0.001$). The HCV group was older and had a lower platelet count and higher ARFI score than the other groups ($P < 0.001$). The NBNC group showed a higher body mass index and platelet count, a higher prevalence of pre-diagnosed non-HCC cancers ($P < 0.001$), especially breast cancer, and a lower prevalence of cirrhosis. Male gender, ARFI score, and HBV were independent predictors of HCC. The 5-year risk of HCC was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis. ARFI alone had an area under the receiver operating characteristic curve (AUROC) of 0.742 for prediction of HCC in 5 years. AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. No difference was found in mortality rate among the groups.

CONCLUSION

The HBV group showed a higher prevalence of HCC but lower comorbidity that made mortality similar among the groups. Those patients with ARFI-graded severe fibrosis or cirrhosis should receive regular surveillance.

Key Words: Non-alcoholic fatty liver disease; Hepatitis B; Hepatocellular carcinoma; Acoustic radiation force impulse; Mortality; Comorbidity

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Core Tip: Among 1962 patients who received an acoustic radiation force impulse (ARFI) study, the 5-year risk of hepatocellular carcinoma (HCC) was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis, respectively. The prevalence of HCC was highest in the hepatitis B virus (HBV) group. However, the HBV group showed the lowest comorbidities among the groups after adjusting for age, gender, and body mass index. This made the mortality rate similar among the groups. ARFI alone had an area under the receiver operating characteristic curve (AUROC) of 0.742 for prediction of HCC in 5 years. The AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. Those patients with ARFI-estimated severe fibrosis or cirrhosis should receive active surveillance of HCC in all etiologies.

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INTRODUCTION

Chronic liver diseases are major risk factors for hepatocellular carcinoma (HCC)[1,2]. Regular screening of high-risk groups to detect HCC in the early stage can increase the chance to eradicate HCC and improve survival rates[3,4]. Liver cirrhosis is a major HCC risk factor[1-4]. In the last two decades, Fibroscan has been used to assess the HCC risk for patients with chronic liver diseases[5-9]. An alternative is the acoustic radiation force impulse (ARFI) imaging which uses ultrasound as a push pulse to measure the liver stiffness[10-12]. Both modalities are quite good in patients with chronic hepatitis C virus (HCV) infection and non-alcoholic liver diseases[13]. However, the correlation was relatively poor in patients with chronic hepatitis B virus (HBV) infection. We have been using ARFI to assess liver stiffness since 2011[12,13], which allowed the informatics department in our institute to produce a uniquely available large-scale cohort of patient data for research purposes. This would facilitate us to evaluate the ability of ARFI to assess the HCC risk using a consecutive cohort that is much larger than what has been found in prior work. The short-term correlation between ARFI and liver fibrosis is relatively poor in HBV, but the long-term effect is still not fully investigated. As well, studies into the predictive ability of ARFI for HCC among patients with different etiologies of chronic liver disease are still rare[14]. Finally, the chronic HBV infection is characterized by an initial immune tolerance phase[15]. This could be a survival strategy[16] despite an increased risk of HCC caused by chronic persistent HBV infection[1,2,17,18]. Therefore, we examined the differences in morbidities and mortalities between HBV and other etiologies in this cohort to help shed some light on this question.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB) of the Chang Gung Medical Foundation (CGMH IRB No. 201801283B0 and No. 202200758B0).

ARFI measurement

ARFI imaging was done with an Acuson S2000 system (Siemens Medical Solutions). Liver stiffness was measured with a standardized protocol at two locations of the right hepatic lobe[12,13]. The mean of the two locations was used as the final measurement. Most of the studies were done by one senior technician (HTW). Because it is not covered by the Taiwanese National Health Insurance Administration, the charge of ARFI (around 50 United States dollars) was mostly paid by the patients. The exceptions were patients undergoing a liver biopsy study or those participating in clinical trials (around 25%), where the cost was paid for by research grants. The first ARFI study was used in this analysis. The ARFI-estimated fibrosis grades was according to the cutoff values in our previous histology proven study[13].

Patients

The data of this study were retrieved from the Chang Gung Research Database of the Chang Gung Memorial Hospital, Linkou and Taipei branches. All patients who received ARFI imaging between January 2011 and September 2018 represented as the index patients. A total population of 2405 patients were included. The Chang Gung Research Database includes the original electronic medical records, comprising health care facility information, patient demographics, diagnosis information, drug information, procedure information, and other health digital information. All the personal identifiers were replaced by a code. We organized a team that included researchers from both hepatology and informatics departments of Chang Gung Memorial Hospital, along with outside technical consultants, to deal with this project[19]. We excluded patients with incomplete viral markers and clinical data, dual viral hepatitis, alcoholic liver diseases, autoimmune liver diseases, toxic hepatitis, genetic liver diseases, and those whose ARFI studies were with an interquartile range over a median ratio > 30%, which is a recommended quality assurance criterion (Figure 1)[20]. According to the viral markers, patients were classified into three groups: Hepatitis B, hepatitis C, and non-hepatitis B, non-hepatitis C (NBNC) patients. The NBNC group consisted mostly of patients with non-alcoholic fatty liver disease[19]. All the patients were followed for liver biochemistry, alpha-fetoprotein, and liver ultrasound at 3-12-mo intervals.

Comorbidity

The comorbidities under investigation included hypertension [I10-I15], type 2 diabetes mellitus [E8-13], dyslipidemia [E78], myocardial infarction [I21-I23, I1252], atrial fibrillation [I48], heart failure [I50], and ischemic stroke [I63-I66]. The ascertainment of these comorbidities was based on three diagnoses from the outpatient department or one diagnosis from the inpatient department.

Antiviral therapy

Patients could have undergone any of several different antiviral regimens in the study period. Similar interferon regimens had been used in both HBV and HCV groups, and such immune modulatory regimens were not uniform. Therefore, we simply recorded whether interferon therapy was given pre-enrollment or post-enrollment.

Similarly, multiple nucleot(s)ide analogue (NA) regimens for HBV and direct antiviral agent (DAA) regimens for HCV were possibly taken in the study period. Their mechanisms are suppression or elimination of viral replication. We recorded such oral therapies in pre-enrollment and/or post-enrollment periods as one category.

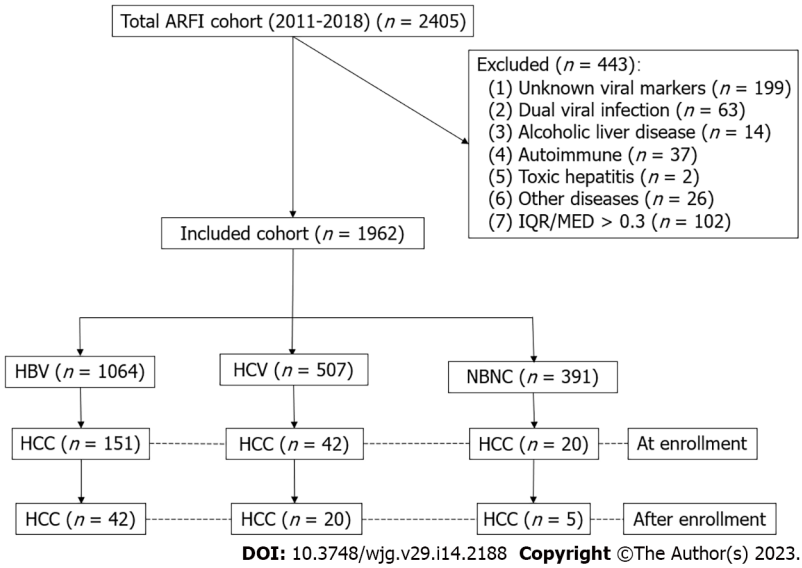
Cancer registration data link

In addition to their medical records, study subjects were linked with the database of Cancer Registration, Chang Gung Memorial Hospital, which records information on all cancers diagnosed in this hospital since 1987. We linked this database up to June 30, 2020.

Mortality data link

This study used the national citizen identification numbers of patients to search the mortality data bank established by the Statistics Office, Department of Health, Taiwan. The mortality data bank stored death certificate data, which includes patient demographic data such as the time, place, and cause of death, and the name of the official who issued the document. Causes of death were classified using the International Classifications of Diseases, Injuries and Causes of Death (ICD-10, World Health Organization, 2015). The data was linked up to December 31, 2020.

This mortality data link will collect data for those patients lost to follow-up in this hospital.



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Figure 1 Patient flow chart. HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, hepatitis C.

Statistical analysis

Patient characteristics are represented as the number and percentage, or the mean ± SD, as appropriate. Continuous variables of the three independent groups were compared using one-way analysis of variance (ANOVA) with Bonferroni correction. Categorical variables were tested using the chi-square test, or the chi-square test for trend, as applicable. Logistic regression was conducted to identify independent risk factors for HCC for records before the ARFI study. Cox proportional hazards model was conducted to identify independent risk factors of HCC for records after the ARFI study. The Mantel-Cox procedure (Log Rank test) was applied to compare risk factors and the cumulative risk of HCC among different groups after the ARFI study. The area under the receiver operating characteristic curve (AUROC) was calculated using a scoring system for HCC risk prediction. The scoring system was based on the hazard ratio in the multivariate analysis. Briefly, ARFI and FIB4 were according to its values and modified by timing 0.5 or 2. Other factors were calculated as: Gender (G) (male = 2, female = 0); etiology score (E) (HBV = 3, HCV = 2, NBNC = 1); age (year) score (A) (0-35 = 0, 35-40 = 1, 40-45 = 2, 45-50 = 3, 50-55 = 4, 55-60 = 5; > 60 = 6; platelet (10^9/L) score (A) (0-100 = 3, 100-150 = 2, > 150 = 1). Statistical analyses were performed using the SPSS software (version 22; SPSS Inc., Chicago, IL, United States), and a P value of < 0.05 was judged as statistically significant.

RESULTS

Baseline demographic features

A total of 1962 patients met our inclusion criteria. Among them, 1064, 507, and 391 patients were in the HBV, HCV, and NBNC groups, respectively (Table 1). The HBV group had more men and a lower prevalence of hypertension, diabetes mellitus, heart failure, ischemic stroke, and dyslipidemia. Patients in the HCV group were older and had a lower male ratio, lower platelet counts, higher fibrosis index based on four factors (FIB4), and mean ARFI score. The NBNC group had a higher body mass index (BMI), lower prothrombin time and international normalized ratios (INR), lower FIB4, higher platelet count, lower rate of estimated cirrhosis, and shorter duration of follow-up.

Alanine aminotransferase level grades at the enrollment

ARFI is strongly influenced by liver inflammation. This especially happens when alanine aminotransferase (ALT) levels are greater than 5 × (180 U/L) the upper limit of normal (0-36). So, we list the ALT level grades in the Supplementary Table 1. In total, 7.3% of patients had ALT level greater than 180 U/L.

Comorbidity

There were significant differences in the prevalence of hypertension, diabetes mellitus, heart failure, ischemic stroke, and dyslipidemia between the HBV group and the other two groups (Table 1). After adjusting for age, gender, and BMI, the prevalence of hypertension, diabetes mellitus, ischemic stroke, and dyslipidemia was significantly lower in the HBV group than in the non-HBV groups (Supplementary Table 2).

Table 1 Baseline demographic features of the cohort

	HBV (*n* = 1064)	HCV (*n* = 507)	NBNC (*n* = 391)	*P* value	Missing rate
Demographics					
Age (yr)	52.05 ± 10.90	58.69 ± 10.84	51.97 ± 13.10	< 0.0001 (2 & 3, 1 & 2)	0.00%
Male sex, *n* (%)	825 (77.5)	273 (53.8)	233 (59.6)	< 0.0001	0.00%
Weight (kg)	68.36 ± 12.30	64.53 ± 12.17	70.94 ± 14.09	< 0.0001 (2 & 3, 1 & 2, 1 & 3)	0.97%
Height (cm)	166.34 ± 7.62	161.67 ± 8.57	164.18 ± 8.75	< 0.0001 (2 & 3, 1 & 2, 1 & 3)	2.80%
BMI (kg/m^2)	24.66 ± 3.62	24.58 ± 3.75	26.25 ± 4.23	< 0.0001 (2 & 3, 1 & 3)	3.47%
Lab data at ARFI study					
Spleen index (cm^2)	31.97 ± 14.47	33.63 ± 16.72	34.07 ± 16.05	0.0640 (1 & 3)	0.03%
Albumin (mg/dL)	4.345 ± 0.51	4.29 ± 0.51	4.43 ± 0.48	0.0040 (2 & 3)	42.15%
AST (U/L)	57.50 ± 102.16	62.46 ± 58.08	58.40 ± 50.72		1.12%
ALT (U/L)	75.05 ± 162.88	71.52 ± 69.61	83.46 ± 78.32		1.33%
Bilirubin (mg/dL)	0.93 ± 1.23	0.91 ± 1.19	0.92 ± 1.21		12.79%
Prothrombin time (INR)	1.10 ± 0.13	1.11 ± 0.21	1.06 ± 0.13	0.0040 (2 & 3, 1 & 3)	42.15%
Platelets (10^9/L)	177.86 ± 61.86	170.13 ± 60.19	218.96 ± 76.08	< 0.0001 (2 & 3, 1 & 3)	13.25%
FIB4	2.454 ± 2.720	3.31 ± 3.04	2.06 ± 2.13	< 0.0001 (2 & 3, 1 & 2)	23.35%
Mean ARFI (m/s)[1]	1.40 ± 0.46	1.60 ± 0.61	1.42 ± 0.62	< 0.0001 (2 & 3, 1 & 2)	0.00%
Stiffness status[1]				< 0.0001	
Cirrhosis	331 (31.1)	163 (32.1)	78 (19.9)		
Severe fibrosis	138 (13.0)	110 (21.7)	61 (15.6)		
Moderate fibrosis	114 (10.7)	68 (13.4)	29 (7.4)		
Mild or non-fibrosis	481(45.2)	166(32.7)	223(57.0)		
Comorbidities					
Hypertension	181 (17.0)	147 (29.0)	115 (29.4)	< 0.0001	
Diabetes	119 (11.2)	103 (20.3)	76 (19.4)	< 0.0001	
Heart failure	14 (1.3)	17 (3.4)	7 (1.8)	0.023	
Atrial fibrillation	9 (0.8)	11 (2.2)	5 (1.3)	0.091	
Myocardial infraction	12 (1.1)	5 (1.0)	2 (0.5)	0.567	
Ischemic stroke	6 (0.6)	17 (3.4)	8 (2.0)	< 0.0001	
Dyslipidemia	170 (16.0)	95 (18.7)	125 (32.0)	< 0.0001	
Follow-up (year)					
mean ± SD	4.59 ± 2.22	4.34 ± 2.35	3.13 ± 2.15	< 0.0001 (1 & 3, 2 & 3)	
Median/IQR	4.54/3.59	3.97/3.88	2.61/3.23		

[1]Cutoff values according to reference 11.

ARFI: Acoustic radiation force impulse; FIB4: Fibrosis-4 index; BMI: Body mass index; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, non-hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Cancers diagnosed before ARFI study

We linked our index cases with cancer registration databases between 1987 and 2020. There were many cancer diagnoses prior to enrollment. These cancers were either well-treated or receiving active therapy. The HBV group had the highest rate of pre-enrollment HCC diagnosis (14.1%, $P < 0.001$, Table 2, upper panel). On the other hand, the NBNC group had the highest rate of non-HCC cancer (10%, $P < 0.001$), with breast cancer having the highest rate (4.3% for total or 10.8% for females alone, $P < 0.001$). Factors associated with a high risk of HCC as identified by logistic regression were male sex, high mean ARFI

Table 2 Cancers and mortality in different groups

	HBV	HCV	NBNC	*P* value
Total cases	1064	507	391	
Female	239 (22.5)	234 (46.2)	158 (40.4)	< 0.027
Follow years before enrollment	-2.65 ± 3.98	-2.78 ± 5.56	-3.30 ± 6.25	< 0.001 (3 & 1, 3 & 2)
Cancers, pre-enrollment				
HCC	150 (14.1)	42 (8.3)	20 (5.1)	< 0.001
Non-HCC cancer	36 (3.4)	30 (5.9)	39 (10.0)	< 0.001
Colon cancer	9 (0.8)	3 (0.6)	6 (1.5)	NS
Breast cancer	1 (0.1)	9 (1.8)	17 (4.3)	< 0.001[1]
Hematology cancers	3 (0.3)	4 (0.8)	2 (0.5)	NS
Follow years before enrollment	-2.76 ± 4.55	-3.57 ± 5.18	-3.22 ± 4.40	NS
Cancers, post-enrollment				
HCC	43 (4.0)	19 (3.7)	5 (1.3)	0.033
Non-HCC tumor	21 (2.2)	14 (2.8)	12 (3.1)	NS
Colon cancer	2 (0.1)	3 (0.6)	1 (0.3)	NS
Breast cancer	2 (0.2)	0 (0.0)	1 (0.3)	NS
Hematology cancers	6 (0.6)	0 (0.0)	1 (0.5)	NS
Prostate cancer	2 (0.1)	1 (0.2)	1 (0.3)	NS
Follow years after enrollment	2.43 ± 1.86	2.51 ± 1.86	2.25 ± 2.00	NS
Mortality total	72 (6.8)	41 (8.1)	21 (5.4)	NS
HCC	35 (3.3)	13 (2.6)	4 (1.0)[2]	0.057
Non-HCC cancers	16 (1.5)	8 (1.6)	7 (1.8)[2]	NS
Liver disease	6 (0.6)	7 (1.4)	6 (1.5)	NS
Non-liver disease	15 (1.4)	13 (2.6)	4 (1.0)	NS
Follow years after enrollment	2.59 ± 1.96	2.69 ± 1.99	2.00 ± 1.87	NS

[1]Female only.
[2]$P = 0.027$.
Remark: Different cancers on the same patient were counted separately. HCC: Hepatocellular carcinoma; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, non-hepatitis C; NS: Not significant.

score, HBV, older age, higher aspartate aminotransferase (AST) level, lower ALT level, and lower platelet count (Table 3). Hypertension and diabetes mellitus were associated with a higher risk of pre-diagnosed HCC while dyslipidemia was associated with a lower risk of pre-diagnosed HCC.

Cancers diagnosed after ARFI

After excluding patients with cancer diagnosed before enrollment, the HBV (4.0%) and NBNC (1.3%) groups exhibited the highest and lowest rates of HCC occurrence, respectively ($P = 0.033$, Table 2, middle panel). Factors associated with HCC diagnosis were male sex ($P = 0.013$), high ARFI score (< 0.001), and HBV ($P = 0.019$, Table 4). In Cox's regression analysis, male gender, HBV, platelet count, and ARFI score were associated with a higher risk of HCC.

HCC development after enrollment in subjects with different fibrosis stages

According to the cutoff values of mean ARFI determined in our previous histology proven cases[13], we divided the study population into cirrhosis, severe fibrosis, moderate fibrosis, and mild to non-fibrosis groups. The cumulative risk of HCC development was highest in the cirrhosis group, followed by the severe fibrosis group, and lowest in the none to moderate fibrosis groups ($P < 0.001$, Figure 2). The 5-year risk of HCC was 5.9% and 9.8% for those patients ARFI-graded with severe fibrosis and cirrhosis, respectively. When those patients with ALT greater than 5 × upper limit normal were removed, the 5-year risk of HCC was 6.1% and 10.4% for those with severe fibrosis and cirrhosis, respectively

Table 3 Logistic regression for hepatocellular carcinoma diagnosed before enrollment

	Univariate analysis	Multivariate analysis		95%CI	
	P value	*P* value	Hazard ratio	Lower	Upper
Male	< 0.001	0.000	3.399	2.150	5.373
Etiology	< 0.001	0.000	-	-	-
HBV		0.000	4.009	2.219	7.241
HCV		0.169	1.644	0.810	3.335
Age, yr	< 0.001	0.000	1.047	1.029	1.066
ALT	0.243	0.001	0.993	0.989	0.997
AST	0.010	0.000	1.012	1.007	1.017
Bilirubin	0.170	0.729	0.980	0.875	1.098
Platelet	0.138	0.035	1.003	1.000	1.005
Spleen index	0.060	0.811	1.001	0.990	1.013
BMI	0.015	0.076	0.956	0.910	1.005
ARFI	< 0.001	0.010	1.556	1.110	2.181
Interferon therapy	0.011	0.012			
Pre-enrollment		0.618	1.204	0.581	2.496
Post-enrollment		0.004	0.211	0.073	0.617
Oral anti-virus agents	0.041	0.181			
Pre-enrollment		0.168	1.721	0.795	3.728
Post-enrollment		0.156	0.667	0.381	1.167
Pre- and post-enrollment		0.527	1.534	0.408	5.773
Hypertension	< 0.001	0.000	2.551	1.702	3.824
Diabetes mellitus	< 0.001	0.031	1.618	1.044	2.508
Dyslipidemia	< 0.001	0.000	0.358	0.207	0.620
Ischemic stroke	0.341	0.884	1.097	0.317	3.794

1481/1962 (75.5%) with complete data; cases (hepatocellular carcinoma), n = 189. HBV: Hepatitis B; HCV: Hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; ARFI: Acoustic radiation force impulse.

(Supplementary Figure 1). There was no difference in predictive abilities across different etiologies (Supplementary Figure 2), suggesting that high ARFI score is a good predictor of HCC diagnosis for HBV patients, along with HCV and NBNC patients.

AUROCs of different non-invasive scoring models for HCC prediction

The AUROCs showed that ARFI or FIB4 alone gave similar 3- or 5-year predictions of HCC (AUROCs around 0.739-0.756) (Figure 3). After adding gender, etiology, age, and platelet score, the AUROCs increased to 0.772-0.840.

Mortality during study period

No significant difference in total mortality was found across the three groups. The HBV group had a marginally higher HCC related mortality (3.3%, P = 0.057) (Table 2, lower panel). However, the non-HCC related mortality was the lowest in the HBV group (P < 0.027). Male sex, age, lower ALT level, higher AST level, lower BMI, higher ARFI score, and ischemic stroke were associated with a higher risk of mortality in Cox's regression analysis (Supplementary Table 3).

DISCUSSION

In our patient cohort, 212 patients were diagnosed with HCC prior to enrollment and 67 developed

Table 4 Cox's regression analysis for hepatocellular carcinoma occurring after enrollment

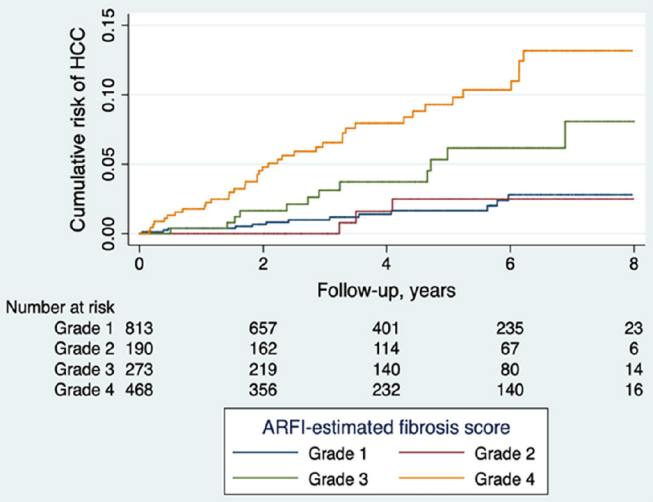
	Univariate analysis	Multivariate analysis		95%CI	
	P value	*P* value	Hazard ratio	Lower	Upper
Male	0.005	0.009	20.796	1.293	6.045
Etiology	0.019	0.057			
HBV		0.023	40.473	1.232	16.245
HCV		0.150	20.670	0.701	10.173
Age (yr)	< 0.001	0.094	10.028	0.995	1.061
ALT (U/L)	0.188	0.211	0.994	0.985	1.003
AST (U/L)	0.605	0.449	10.005	0.993	1.017
Bilirubin (mg/dL)	0.962	0.326	0.772	0.460	1.295
Platelet (10^9/L)	< 0.001	0.041	0.993	0.986	1.000
Spleen index (cm^2)	0.019	0.169	10.012	0.995	1.030
BMI	0.334	0.524	0.973	0.894	1.059
ARFI (m/s)	< 0.001	0.000	20.775	10.624	4.742
Interferon therapy	0.814	0.943			
Pre-enrollment		0.734	0.772	0.174	3.435
Post-enrollment		0.914	0.949	0.366	2.459
Oral anti-virus therapy	0.0174	0.086			
Pre-enrollment		0.977	10.031	0.134	7.948
Post-enrollment		0.029	20.105	10.080	4.102
Pre- and post-enrollment		0.110	30.362	0.758	14.903
Hypertension	0.001	0.211	10.554	0.778	3.104
Diabetes mellitus	0.002	0.304	10.460	0.710	3.006
Ischemic stroke	0.305	0.971	00.000	0.000	1.46E+269
Dyslipidemia	0.233	0.065	10.920	0.960	3.840

1274/1749 (72.8%) with complete data, 49 cases (hepatocellular carcinoma). HBV: Hepatitis B; HCV: Hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; ARFI: Acoustic radiation force impulse.

HCC after enrollment (Table 2). High ARFI scores were associated with pre-diagnosed HCC occurrences in a multivariate analysis ($P < 0.001$, Table 3) and were a good predictor of HCC after enrollment according to Cox's regression analysis ($P < 0.001$, Table 4). The ARFI-estimated fibrosis grades showed a severity-dependent increased risk of HCC in the post-enrollment period (Figure 2). The 5-year risk of HCC was 5.9% and 9.8% for those patients who were ARFI-graded with severe fibrosis and cirrhosis, respectively. When age, gender, and platelet count were added, there was only a small rise of AUROC from 0.753 to 0.84 within 3 years and 0.742 to 0.828 within 5 years in the prediction of HCC. These results suggest that liver fibrosis is the main risk factor for HCC (Figure 3). Our finding is consistent with others in a recent study review[21] using non-invasive fibrosis diagnosis models. Patients with grades 3 and 4 fibrosis should receive active surveillance of HCC.

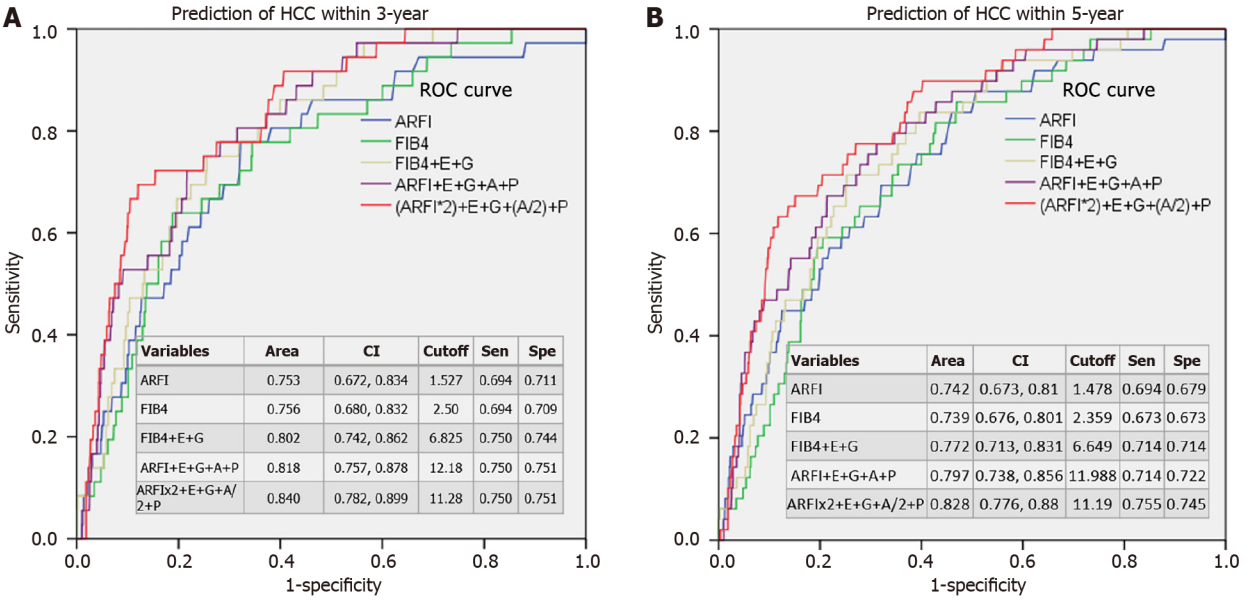
ARFI-graded fibrosis predicts the HCC occurrence well among different etiologies (Supplementary Figure 2). Although the correlation between ARFI and liver histology fibrosis was poorer in the HBV group[13] than in the other two etiologies, HCC risk prediction was as good as that in the other groups. This question was not well-addressed in prior work where such investigations had much smaller clinical datasets or single etiology cohorts. Additional investigations should further pursue this link.

In terms of prior work, ARFI studies on non-HCC patients typically focused on its utility of fibrosis staging[10-12,22]. Exceptions include the work by Sun *et al*[23], who correlated ARFI with the indocyanine green test and found a positive correlation between the two. ARFI was also correlated to Child-Pugh scores in that study. They suggested that ARFI imaging is a useful tool for assessing liver functional reserve. As another example, a recent meta-analysis reported that ARFI scores may be a good



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Figure 2 Cumulative risk of hepatocellular carcinoma after enrollment in different acoustic radiation force impulse-fibrosis grades. Higher risk of hepatocellular carcinoma (HCC) was found in acoustic radiation force impulse (ARFI)-severe fibrosis and -cirrhosis grades than in none to moderate fibrosis grades. The 5-year risk of HCC was 9.8 % for ARFI fibrosis graded as cirrhosis; 5.9% for that graded as severe fibrosis; and only 1.7%-2.0% for that lower or equal to moderate fibrosis. Purple: Cirrhosis; Yellow: Severe fibrosis; Green: Moderate fibrosis, Blue: None or mild fibrosis [Log rank test: $P = 0.026$ (3 *vs* 4); $P = 0.015$ (3 *vs* 1 + 2); $P < 0.001$ (4 *vs* 1 + 2)]. HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse.



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Figure 3 Area under the receiver operating characteristic curve of hepatocellular carcinoma prediction in different non-invasive scoring models. A: Within 3-year period after enrollment; B: Within 5-year period after enrollment. The area under the receiver operating characteristic curve (AUROC) shows that acoustic radiation force impulse (ARFI) or FIB4 alone give similar 3- or 5-year predictions of hepatocellular carcinoma (HCC) (AUROC around 0.739-0.756). After adding G (gender score), E (etiology score), A (age score), and P (platelet score), the AUROC may increase to 0.772-0.840. Both ARFI and FIB4 models predict 3- or 5-year HCC quite satisfactorily, suggesting that fibrosis is the main risk factor for HCC. ARFI: Acoustic radiation force impulse; FIB4: Fibrosis index based on four factors; G: Gender score, male = 2, female = 0; E: Etiology score, hepatitis B virus (HBV) = 3, HCV = 2, NBNC = 1; A: Age score (year), 0-35 = 0, 35-40 = 1, 40-45 = 2, 45-50 = 3, 50-55 = 4, 55-60 = 5; > 60 = 6; P: Platelet score (10^9), 0-100 = 3, 100-150 = 2, > 150 = 1; Sen: Sensitivity; Spe: specificity; HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse; FIB4: Fibrosis-4 index.

predictor of HCC recurrence-free survival in patients receiving radiofrequency ablation[24]. In a series of 1808 patients who received ARFI, those patients with an ARFI score > 1.33 cm/s showed a higher probability of HCC development than those with an ARFI score ≤ 1.33 cm/s[25]. Therefore, there is good evidence that ARFI can measure liver fibrosis, reflex the liver functional reserve, and predict HCC recurrence. Our study adds to these conclusions, as Cox's regression analysis confirmed that high ARFI score was a risk factor for HCC and mortality ($P < 0.001$, Table 3 and Supplementary Table 3).

In aspects of HCC diagnosis, the HBV group had higher occurrences than the NBNC group (Table 2). This aligns with previous investigations, *e.g.*, the study by Chen *et al*[26] who reported higher occurrences of HCC in their HBV (4.8%) and HCV (4.7%) groups compared to their NBNC group (0.3%). Even so, the prevalence of HCC in our NBNC group was much higher than their value, but this is because Chen *et al*[26] included healthy subjects in their NBNC group, while our NBNC cohort was a disease group. Even though NBNC patients had a lower incidence of HCC ($P < 0.001$), this was offset by higher incidences of non-HCC cancers. Consequently, the total mortality rate was similar among different groups (Table 2). This is consistent with a recent meta-analysis by Mantovani *et al*[27] that concluded that extra-hepatic cancers were increased in non-alcoholic fatty liver disease. It should be noted that such trends were only identified in the pre-enrollment period because it had a longer past history. We can record many cancers in the pre-enrollment period because the diagnosis of all cancer types has been recorded in our cancer registration database since 1987. We found that breast cancer ($P < 0.001$), colon cancer, and hematologic cancers were the main cancers in the pre-enrollment period. These types of cancers respond to treatment well, allowing us to include such survivors in our cohort.

There were lower rates of comorbidities in the HBV group than in the other groups (Table 1). This seems to be related to the high prevalence of metabolic syndrome in the HCV or NBNC group. However, after adjusting for gender, age, and BMI, such a phenomenon was still present (Supplementary Table 2). As early as 2006, Jan *et al*[28] had reported a lower prevalence of diabetes, hypertension, obesity, hyperlipidemia, and obesity in HBV than HCV or NBNC in a population-based study in northern Taiwan. Another nationwide study by Kuo *et al*[29] that examined 1376344 diabetes patients between 2000 and 2012 also confirms these results. Before enrollment, they excluded patients with a history of myocardial infarction (2.28% in HBV group and 4.19% in non-HBV group, $P < 0.001$) and cerebrovascular diseases (15.6% in HBV group and 24.3% in non-HBV group, $P < 0.001$). The percentage of excluded patients was lower in their HBV group than the non-HBV groups for both diseases. After enrollment and propensity matching, the risk of all-cause mortalities, myocardial infarction, ischemic stroke, and heart failure was higher in the non-HBV group than in the HBV group ($P < 0.001$) in a mean follow-up period of 5.3 year[29]. A study by Sung *et al*[30] from Korea showed similar findings. They pointed out that the difference was more profound in HBV with liver dysfunction than in those without liver dysfunction. They suggested that the HBV-related proinflammatory effect may be the reason for the decreased risk of comorbidity.

Previous studies did not discuss the reason for these low comorbidities in the HBV patients. A recent review suggested that chronic HBV infection may protect infected subjects from the development of metabolic syndrome and hepatic steatosis[31]. The immune system is a double-edged sword. Its efforts against microorganisms may induce host tissue damage[32]. Chronic persistent HBV infection is characterized by an initial immune tolerance phase that allows active HBV replication without immune-mediated inflammation to liver tissue[33]. Liver inflammation occurs only when the immune system is triggered to attack HBV carrying hepatocytes[34]. This contrasts with HCV and NBNC groups with persistently mild inflammation in the liver.

There is a longer HBV-related immune tolerance phase in East Asian than in African, which could be related to genetic polymorphism in human leukocyte antigen (HLA)-DP and -DQ loci[16,35]. Such HBV-related gene variants not only decrease antigen presentation to avoid fatal immune response, but also establish an environment that is suitable for chronic persistent HBV infection[16]. Our recent study indicated that those HBV-related single nucleotide polymorphisms (SNPs) in HLA-DP and -DQ loci were associated with high viral load in the HCC family[36]. Such patients would be more likely to be associated with liver dysfunction as those mentioned in Sung's series[30]. From the above clues, we suspect that the low comorbidity trend in the HBV group may be partly associated with a low antigen presentation ability.

One of our limitations is that life-long disease consequences are not easy to examine. The mean follow-up duration after enrollment could be considered as relatively short (3.13 to 4.59 years). We may need longer and more specific studies to explore the link between HBV infection and comorbidity. It should be noted that HBV-related SNPs in HLA-DP and -DQ loci in East Asians are quite different from other ethnicities[16]. The low comorbidity in the HBV group may be limited to East Asian. Another limitation is that the contribution of therapy to morbidity and mortality was difficult to evaluate. We noticed that the post-enrollment interferon therapy was associated with a lower prevalence of pretreatment HCC and post-enrollment oral antivirus therapy was associated with a lower risk of post-enrollment HCC. However, these findings may be due to a relatively better condition of chronic liver disease, which could have made the pre-enrollment therapy unnecessary. Similar situations concerning about therapeutic response in HCC-related mortality could be presented. With the success of checkpoint inhibitors in HCC therapy[37], future predictive biomarker study will be needed to clarify the difference in mortality among groups[38].

CONCLUSION

We conclude that even ARFI-based fibrosis prediction in the HBV group is poorer than that in other

groups, its performance or clinical significance in predicting HCC or mortality is as good as that in other etiologies. The HBV group had the highest risk of HCC and the NBNC group had the highest risk of non-HCC tumors, especially breast cancer. Low prevalence of comorbidities in the HBV group was found, which may be a consequence of a low prevalence of metabolic syndrome and low antigen presentation ability.

ARTICLE HIGHLIGHTS

Research background

Acoustic radiation force impulse is used to measure liver fibrosis and predict outcomes. The performance of elastography in assessment of fibrosis is poorer in hepatitis B virus (HBV) than in other etiologies of chronic liver disease.

Research motivation

Whether there are differences in performance of acoustic radiation force impulse (ARFI) in long term outcome prediction among different etiologiesof chronic liver disease remains to be studied.

Research objectives

We collected a cohort of patients who received ARFI studies. After excluding unsuitable cases, 1962 patients were included as the indexed cases. They were classified into HBV, HCV, and non-HBV, non-HCV (NBNC) groups. We examined the differences in demographics, comorbidity, carcinogenesis, and mortality among these groups at and after enrollment.

Research methods

These indexed cases were linked to the hospital's cancer registration and national mortality databases to obtain complete outcome data. The data at enrollment were analyzed for differences among three groups and logistic regression was performed to search for predictors associated with cancers. Cox regression analysis and area under the receiver operating characteristic curve (AUROC) were used to assess the performance of ARFI in predicting hepatocellular carcinoma (HCC) and mortality.

Research results

At enrollment, the HBV group showed more males (77.5%), a higher prevalence of pre-diagnosed HCC, and a lower prevalence of comorbidities than the other groups ($P < 0.001$). The HCV group was older and had a lower platelet count and higher ARFI score than the other groups ($P < 0.001$). The NBNC group showed a higher body mass index, platelet count, and prevalence of pre-diagnosed non-HCC cancers ($P < 0.001$), especially breast cancer, and a lower prevalence of cirrhosis. After enrollment, male gender, ARFI score, and HBV were independent predictors of HCC. The 5-year risk of HCC was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis, respectively. ARFI alone had an AUROC of 0.742 for prediction of HCC in 5 years. AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. No difference in mortality rate was noted among the groups.

Research conclusions

The HBV group showed a higher prevalence of HCC but a lower prevalence of comorbidity that made mortality similar among the groups. Those ARFI-graded as severe fibrosis or cirrhosis should receive regular surveillance.

Research perspectives

The immune tolerance is a hallmark of HBV which could be related to poor antigen presentation of human leukocyte antigen-DP and -DQ molecules in HBV surface antigen carriers. Whether such behavior is associated with a low prevalence of comorbidity requires future study.

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FOOTNOTES

Author contributions: Tai DI contributed to study concept and design; Tai J, Chen HM, Hsu CY, Chen CJ, Jeng WJ, Chang ML, and Tai DI contributed to data acquisition; Tai J, Harrison AP, Chen HM, Hsu CY, Jeng WJ, Chang ML, and Tai DI contributed to data analysis and interpretation; Tai J, Harrison AP, and Tai DI contributed to drafting of the manuscript; Harrison AP, Jeng WJ, Chang ML, and Lu L contributed to critical review of the manuscript for important intellectual content; Tai J, Chen HM, and Hsu CY contributed to statistical analysis; Tai DI and Lu L contributed to obtaining funding; Chen HM and Hsu CY contributed to technical or material support; Tai DI contributed to study supervision.

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Informed consent statement: The Institutional Review Board of the Chang Gung Medical Foundation waived the requirement of informed consent statement for this retrospective cohort study.

Conflict-of-interest statement: There is no conflict of interest to disclose.

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SYSTEMATIC REVIEWS

Effectiveness of *Helicobacter pylori* eradication in the treatment of early-stage gastric mucosa-associated lymphoid tissue lymphoma: An up-to-date meta-analysis

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Abstract

BACKGROUND

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. Clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML. To determine the practical effect of bacterial eradication as the sole initial therapy for early-stage GML, an updated analysis and review of available evidence is imperative.

AIM

To perform a meta-analysis to assess the rate of complete remission (CR) of *H. pylori*-positive early-stage GML following bacterial eradication.

METHODS

We performed independent, computer-assisted literature searches using the PubMed/MEDLINE, Embase, and Cochrane Central databases through September 2022. Prospective and retrospective observational studies evaluating the CR of early-stage GML following bacterial eradication in *H. pylori*-positive patients. The risk of bias was assessed using Joanna Briggs Institute (JBI) Critical Appraisal Tools. The pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI) were calculated following the random-effects model. Heterogeneity and inconsistency were assessed using Cochran's *Q* test and *I2* statistic, and heterogeneity was defined as $P < 0.01$ and $I^2 > 50\%$, respectively. Subgroup and meta-regression analyses were conducted to explore potential sources of heterogeneity.

RESULTS

The titles and abstracts of 1576 studies were screened; 96 articles were retrieved and selected for full-text reading. Finally, 61 studies were included in the proportional meta-analysis (P-MA). Forty-six were prospective and fifteen were retrospective uncontrolled, single-arm, observational studies. The overall risk of bias was low to moderate in all but a single report, with an average critical appraisal score across all studies of 79.02%. A total of 2936 *H. pylori*-positive early-stage GML patients, in whom *H. pylori* was successfully eradicated, were included in the analysis. The pooled CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). P-MA indicated the substantial heterogeneity in CR reported across studies (I^2 = 92%; $P < 0.01$). Meta-regression analysis identified statistically significant effect modifiers, including the proportion of patients with t(11;18)(q21;q21)-positive GML and the risk of bias in each study.

CONCLUSION

Comprehensive synthesis of available evidence suggests that *H. pylori* eradication is effective as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the interpretation of the pooled overall CR, the present study is a relevant to informing clinical practice.

Key Words: Lymphoma; B-cell; Marginal zone; Gastric mucosa-associated lymphoid tissue lymphoma; Stomach lymphoma; *Helicobacter pylori*; Therapeutics; Eradication therapy

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Core Tip: Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. Clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML. Despite advances in determining the practical effect of bacterial eradication as sole initial therapy for early-stage GML, an updated meta-analysis of available evidence is imperative. We performed a systematic review with proportional meta-analysis to assess the complete remission rate of *H. pylori*-positive early-stage GML after eradication therapy.

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INTRODUCTION

Marginal zone lymphomas (MZLs) are the third most common type of non-Hodgkin B-cell lymphoma following diffuse large B-cell lymphoma and follicular lymphoma[1]. The 5th edition of the World Health Organization Classification of Hematolymphoid Tumors - Lymphoid Neoplasms subdivides MZL into 4 subtypes: Extranodal MZL of mucosa-associated lymphoid tissue (MALT), primary cutaneous MZL, nodal MZL, and pediatric MZL[2].

Gastric MALT lymphoma (GML) is a low-grade B-cell neoplasia commonly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis[3]. GML provides the best-characterized model of the antigen-induced transition from normal to malignant marginal-zone B-cells[4]. Despite the lack of lymphoid follicles in the normal gastric mucosa, MALT may appear as a result of inflammation. *H. pylori* chronic gastritis induces specific T helper cells and the subsequent expansion of polyclonal B cells, which can undergo malignant transformation[4,5]. Similar to that of gastric cancer, in advanced-stage GML, inflammatory signaling pathway and pro-oncogenic genetic changes allow a microenvironment-independent progression of the tumor, characterizing a "hit-and-run" mechanism[5,6]. The overwhelming evidence suggesting a causal relationship between *H. pylori* infection and GML is also supported by epidemiological data[7].

Although robust comparative studies such as randomized clinical trials have not been carried out, clinical practice guidelines currently recommend *H. pylori* eradication as the sole initial treatment for early-stage GML[8]. Triple-therapy, which comprises a proton pump inhibitor (PPI) for 4 wk and clarithromycin with either amoxicillin or metronidazole for 10-14 d, remains standard. However, given the increasing rate of bacterial clarithromycin resistance in many countries, international guidelines also recommend bismuth quadruple therapy (BQT) or concomitant non-BQT as possible alternatives[9-11]. Accordingly, a previous systematic review with pooled data analyses highlighted that, after a long-term follow-up period, lymphoma disappeared in more than 75% of low-grade, stage I or II_1 gastric lymphoma patients treated with bacterial eradication[12]. This study also identified that when the neoplastic lesion is confined to the submucosa, the main lesion is localized in the distal stomach, and t(11;18)(q21;q21) translocation is absent, the effectiveness of *H. pylori* eradication is even greater.

Given the low incidence of GML and the small sample sizes and heterogeneity of available studies [12], there is a need for an updated statistical analysis of the current evidence regarding *H. pylori* eradication as the sole initial therapy. Here, we performed a systematic literature review with meta-analysis to assess the complete histopathologic remission rate of *H. pylori*-positive early-stage GML after bacterial eradication therapy.

MATERIALS AND METHODS

This study followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guideline, which consists of a 27-item checklist and a 3-phase flowchart. The checklist includes items considered critical to the transparent reporting of a systematic review[13].

Literature search

The search strategy was designed following the Joanna Briggs Institute (JBI) Manual for Evidence Synthesis (https://synthesismanual.jbi.global). We performed independent, computer-assisted searches of the PubMed/MEDLINE, Embase, and Cochrane Central databases for studies published before September 2022. Medical Subject Headings and Embase Subject Headings (Emtree) index terms and free-text words were combined. Search terms included "Lymphoma, B-Cell, Marginal Zone," "Mucosa-Associated Lymphoid Tissue Lymphoma," "Marginal Zone B-Cell Lymphoma," "MALT lymphoma," "Stomach lymphoma," "Helicobacter pylori," "Therapeutics," and "Eradication therapy." Boolean operators (AND, OR) were also used to narrow or broaden the search as required. All citations were exported to the Rayyan (https://www.rayyan.ai/) tool and all duplicates were removed.

Study selection

Two researchers independently assessed the articles according to predefined eligibility criteria. In the case of disagreement, a 3rd researcher was consulted. The titles and abstracts of the articles were analyzed and studies that did not fit the inclusion criteria were excluded. The full texts were then revised to select eligible studies for meta-analysis.

Studies that met the following criteria were included: (1) Prospective and retrospective observational studies (cohort, case-control, and case series) evaluating the complete remission (CR) rate of early-stage GML after bacterial eradication therapy in *H. pylori*-positive patients; and (2) Studies including *H. pylori*-positive patients exclusively treated with antibiotic eradication therapy. Also, only trials enrolling patients with either stage I or II_1 GML according to Lugano classification were included[14].

Exclusion criteria were as follows: (1) Studies that did not report the CR rate of *H. pylori*-positive early-stage GML after bacterial eradication; (2) Studies investigating high-grade or diffuse large B cell lymphomas, except for those where it is possible to extrapolate data from a subgroup with early-stage GML; (3) Studies that included patients with non-gastric sites of MALT lymphoma or ineligible study subjects, such as animals or children; (4) Full-text article not available or article not available in English; (5) Case reports, reviews, meta-analyses, systematic reviews, editorials, conference abstracts; and (6) Studies with insufficient data regarding treatment outcome.

Risk of bias assessment

Two researchers independently assessed the risk of bias using the JBI checklists for cohort, case-control, and case series studies[15]. In cases of disagreement, a 3rd researcher was consulted. These tools include multiple questions to assess the methodological quality of a study and determine the extent to which a study has addressed the possibility of bias in its design, conduct, and analysis. The bias percentage risk was calculated by the number of "yes" (Y) answers selected in the checklist. Questions with "not applicable" (N/A) answers were not considered in the calculation. The risk of bias was classified using the following categories: High (scores up to 49.0%), moderate (scores between 50.0% and 70.0%), and low (scores above 70.0%).

Data extraction

Two investigators extracted data from the selected studies using a predefined data extraction worksheet. Any discrepancies were resolved by a 3rd reviewer. The primary outcome was the complete histopathologic remission of the lymphoma after bacterial eradication in *H. pylori*-positive early-stage GML patients. Data were extracted with respect to the following: (1) Included study-related information (1st author, year of publication, country of origin, study design, and study size); (2) Clinical characteristics of the study population (disease stage, diagnostic methods for *H. pylori* infection, and eradication schemes); (3) Number of *H. pylori*-positive early-stage GML patients treated only with bacterial eradication; (4) Number of patients in whom *H. pylori* was successfully eradicated (either provided or calculated); and (5) Number of patients who finally achieved complete remission of the lymphoma (either provided or calculated). The stage of the lymphoma was assessed using the Lugano classification system[13].

Statistical analysis

The pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI) were calculated following the random-effects model. Forest plots were used to summarize the results. Heterogeneity and inconsistency were assessed using Cochran's Q test and I^2 statistic[16]; heterogeneity was defined as $P < 0.01$ and $I^2 > 50\%$, respectively. A subgroup analysis by study design (prospective; retrospective) was conducted to create more homogenous groups. Furthermore, a meta-regression analysis was conducted to explore potential sources of heterogeneity, such as publication year (≤ 2015; > 2015), geographic region of the study (Asian; Western), the prevalence of the translocation t(11;18)(q21; q21), and risk of bias (low; moderate; high). Analysis of publication bias was not performed as this measure is inappropriate for proportional meta-analysis (P-MA)[17]. All analyses were performed using R software version 4.2.1 (R: A Language and Environment for Statistical Computing, Vienna, Austria), using the 'Meta' package, version 5.2-0.

RESULTS

Literature search and study selection

Figure 1 depicts the flow of information through the different phases of the systematic review. Database searches identified 2375 reports, and duplicates were removed. The titles and abstracts of 1576 studies were screened and 96 articles were retrieved and selected for full-text reading. Finally, 61 studies were included in the meta-analysis. Reasons for exclusion were as follows: (1) 10 reports did not consider different stages in CR calculation; (2) 8 had insufficient data on *H. pylori* infection status; (3) 6 were conference abstracts; (4) 5 were publications of the same investigator or group; (5) 4 had insufficient data on the outcome; and (6) 2 included ineligible study subjects.

Study characteristics

Table 1 summarizes the characteristics of the studies included in the P-MA. The included reports were prospective and retrospective observational studies published between 1993 and 2021. A sample of 3315 patients with early-stage GML was obtained, of which 3003 were *H. pylori*-positive. A total of 2936 patients in whom *H. pylori* was successfully eradicated were included in the analysis. Twenty-nine of the included studies were conducted in Asian countries and 32 in Western countries. Concerning study design, 46 were prospective and 15 were retrospective uncontrolled, single-arm, observational studies. The median number of *H. pylori*-eradicated early-stage GML patients was 38 (ranging from 6-193). Multiple diagnostic tests for *H. pylori* infection and eradication were used, including histologic examination, *H. pylori* culture, rapid urease testing, 13C- or 14C-urea breath testing, serology, and *H. pylori* antigen stool testing. In most studies, at least 2 diagnostic tools were used to determine *H. pylori* infection status. Also in most studies eradication therapy consisted of a combination of 2 antibiotics, such as amoxicillin and clarithromycin, with a PPI. However, dual and quadruple therapies (2 antibiotics + PPI + bismuth or 3 antibiotics + PPI, respectively) were also used. Treatment duration ranged from 7 d to 21 d (Table 2).

Table 1 Characteristics of the included studies

Ref.	Country	Design	Study period	Study population				
				Early-stage gastric MALT lymphoma, *n*	Lugano stage	Median follow-up in mo	*H. pylori*-positive early-stage GML, *n*	Diagnosis of *H. pylori* infection
Yang *et al*[18], 2021	China	Retrospective	2003-2015	70	70	30	52	UBT; HE
Schmelz *et al*[19], 2019	Germany	Prospective	2001-2010	109	109	12	99	HE
Sugizaki *et al*[20], 2018	Japan	Prospective	2010-2016	97	97	37.4	97	HE; HpC; RUT; UBT; HpSA; S
Song *et al*[21], 2018	China	Prospective	2000-2013	122	122	38	47	RUT; HE; UBT
Li *et al*[22], 2016	China	Retrospective	2001-2013	75	75	62.9	69	HE; UBT
Kim *et al*[24], 2016	Korea	Retrospective	2001-2014	49	49	51	40	HE; UBT; RUT
Moleiro *et al*[23], *2016*	Korea	Retrospective	2005-2014	103	103	50.5	82	RUT; UBT; HE
Park *et al*[25], 2016	Portugal	Retrospective	1993-2013	103	103	105	87	HE; HpC; S; UBT
Grgov *et al*[26], 2015	Serbia	Prospective	2002-2012	20	20	NR	20	RUT; HE
Nonaka *et al*[27], 2014	Japan	Retrospective	2007-2012	16	16	NR	12	HE; S; UBT
Lima *et al*[28], 2014	Brazil	Prospective	2009-2010	8	8	24	7	RUT; HE; UBT
Wündisch *et al*[29], 2012	Germany	Prospective	1993-1999	120	120	122	120	HE
Choi *et al*[30], 2011	Korea	Retrospective	2003-2010	35	35	21.5	26	HE; RUT; UBT
Ono *et al*[31], 2011	Japan	Retrospective	2003-2009	21	21	1	13	RUT; UBT; HpC; HE; S
Andriani *et al*[32], 2009	Italy	Retrospective	1993-2006	60	60	65	60	HE
Sumida *et al*[33], 2009	Japan	Prospective	1997-2007	66	66	40	57	HE; S; UBT
Stathis *et al*[34], 2009	Switzerland	Retrospective	1990-2006	105	105	75.6	85	HE; S; UBT
Terai *et al*[35], 2008	Japan	Prospective	1995-2006	74	74	46	70	RUT; HE; S; UBT
Fischbach *et al*[36], 2007	Germany	Retrospective	NR	108	108	42.2	108	HE; UBT
Kim *et al*[37], 2007	Korea	Prospective	1996-2006	99	99	41	99	HE; RUT
El-Zahabi *et al*[38], 2007	Lebanon	Retrospective	1999-2005	22	22	12	19	HE; S
Hong *et al*[39], 2006	Korea	Prospective	1996-2003	90	90	45	90	HE; RUT; UBT
Wündisch *et al*[40], 2006	Germany	Retrospective	1993-2003	196	196	27	196	HE
Akamatsu *et al*[41], 2006	Japan	Prospective	1993-2006	55	55	37.3	38	HpC; HE
Wündisch *et al*[42], 2005	Germany	Prospective	NR	120	120	75	120	HE
Montalban *et al*[43], 2005	Spain	Prospective	1993-2002	24	24	64	24	HE; UBT
Chen *et al*[44], 2005	Taiwan	Prospective	1996-1999	34	34	70	31	HE; RUT; S

Taji *et al*[45], 2005	Japan	Prospective	1995-2001	13	13	32.5	12	HE; HpC; S; UBT; RUT
Takenaka *et al*[46], 2004	Japan	Prospective	1995-2002	33	33	5	33	HpC; RUT
Fischbach *et al*[47], 2004	Germany	Prospective	NR	90	90	44.6	80	RUT; HE; UBT
Sheu *et al*[48], 2003	Taiwan	Prospective	NR	15	15	NR	15	RUT; HE
Caletti *et al*[49], 2002	Italy	Prospective	1997-1999	51	51	24	51	HE; RUT; S
Nakamura *et al* [50], 2002	Japan	Prospective	1994-2001	21	21	14.5	17	HpC; S
Liu *et al*[51], 2002	France; Netherlands; Italy; Germany; England	Retrospective	NR	111	111	NR	111	HE; HpC
Bertoni *et al*[52], 2002	England; Italy; Switzerland	Prospective	NR	62	62	24	62	HE; S
Ohashi *et al*[53], 2002	Japan	Prospective	NR	13	13	NR	13	RUT; HE; HpC
Kim *et al*[54], 2002	Korea	Prospective	NR	20	20	18.3	20	RUT; HE
Matsushima *et al* [55], 2002	Japan	Prospective	1995-1997	14	14	27.5	14	RUT; HE; HpC; UBT
Kanda *et al*[56], 2001	Japan	Prospective	1994-1999	13	13	7	13	HE
Raderer *et al*[57], 2001	Austria	Retrospective	1997-1999	22	22	25	22	HE
Nakamura *et al* [58], 2001	Japan	Prospective	1994-1998	41	41	20.5	41	HpC; S; HE
Ruskoné-Fourmestraux *et al* [59], 2001	France	Prospective	1995-1998	44	44	35	34	HE; HpC; S; PCR
Thiede *et al*[60], 2001	Germany	Prospective	NR	97	97	20.8	97	NR
de Jong *et al*[61], 2001	Netherlands	Prospective	NR	23	23	37	23	HE; HpC
Urakami *et al*[62], 2000	Japan	Prospective	NR	47	47	20	47	RUT; HE; HpC
Papa *et al*[63], 2000	Italy	Prospective	1995-1999	7	7	48	7	HE; UBT
Yamashita *et al* [64], 2000	Japan	Prospective	NR	21	21	NR	21	HE; RUT; HpC
Ohashi *et al*[65], 2000	Japan	Prospective	NR	11	11	NR	11	RUT; HE; HpC
Nakamura *et al* [66], 2000	Japan	Prospective	1993-1998	30	30	NR	26	HpC
Savio *et al*[67], 2000	Italy	Prospective	1991-1997	76	76	NR	76	HE
Weston *et al*[68], 1999	United States	Prospective	NR	68	68	NR	65	HE
Steinbach *et al*[69], 1999	United States	Prospective	NR	34	34	NR	28	HE; RUT; S
Nobre-Leitão *et al* [70], 1998	Portugal	Prospective	NR	17	17	12	17	HE; HpC
Thiede *et al*[71], 1997	Germany	Prospective	NR	84	84	NR	84	NR
Sackmann *et al* [72], 1997	Germany	Prospective	NR	22	22	10	22	HE; HpC
Neubauer *et al*	Germany	Prospective	NR	50	50	24	50	HE

[73], 1997								
Pinotti *et al*[74], 1997	Italy; Switzerland	Prospective	1986-1995	86	86	23.3	45	HE; S
Savio *et al*[75], 1996	Italy; England	Prospective	1991-1993	13	13	NR	13	HE
Bayerdörffer *et al* [76], 1995	Germany	Prospective	NR	33	33	12.5	33	HE
Roggero *et al*[77], 1995	Switzerland; Italy	Prospective	NR	26	26	12	26	HE
Wotherspoon *et al* [78], 1993	England; Italy	Prospective	NR	6	6	NR	6	HE

CR: Complete remission rate; GML: Gastric mucosa-associated lymphoid tissue lymphoma; *H. pylori*: *Helicobacter pylori*; HE: Histologic examination; HpC: *Helicobacter pylori* culture; HpSA: *Helicobacter pylori* stool antigen; MALT: Mucosa-associated lymphoid tissue; NR: Not reported; PPI: Proton pump inhibitor; RUT: Rapid urease test; S: Serology; UBT: 13C- or 14C-urea breath test.

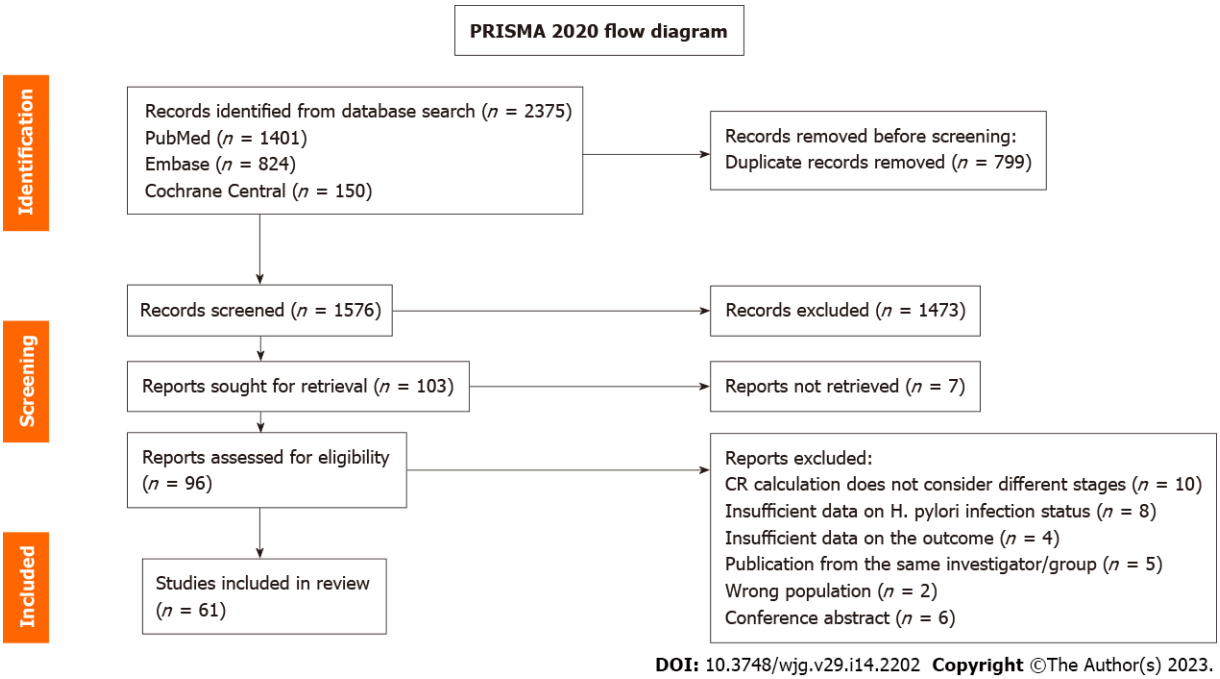


Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-analyses 2020 flow diagram. The flow chart describes the flow of information through the different stages of the systematic review and maps the number of records identified, included, and excluded, and the reasons for study exclusion. *H. pylori*: *Helicobacter pylori*.

Risk of bias in studies

Risk of bias was assessed using JBI checklists (Figure 2). The included single-arm uncontrolled observational studies were classifiable and assessed as case series. The overall risk of bias was low to moderate in all but 1 study[36], with an average critical appraisal score across all studies of 79.02% (Figure 2A).

An increased risk of bias was due to "No" or "Unclear" answers to the following questions: (1) Was there clear reporting of the presenting site(s)/clinic(s) demographic information? (54/61 studies); (2) Did the case series have consecutive inclusion of participants (24/61 studies); (3) Did the case series have complete inclusion of participants? (22/61 studies); (4) Was there clear reporting of the demographics of the participants in the study? (6/61 studies); (5) Was statistical analysis appropriate? (6/61 studies); (6) Was the condition measured in a standard, reliable way for all participants included in the case series? (4/61 studies); (7) Was there clear reporting of clinical information of the participants? (4/61 studies); (8) Was there clear reporting of clinical information of the participants? (3/61 studies); (9) Were the outcomes or follow-up results of cases clearly reported? (8/61 studies); and (10) Were there clear criteria for inclusion in the case series? (2/61 studies). Figure 2B shows the discriminated assessments for each question across all studies.

Table 2 Characteristics of studies reporting the complete remission rate of *Helicobacter pylori*-positive early-stage gastric mucosa-associated lymphoid tissue lymphoma after bacterial eradication

Ref.	Region	*H. pylori*-positive early-stage gastric MALT lymphoma, *n*	*H. pylori*-eradicated gastric MALT lymphoma patients, *n*	CR, *n*	t(11;18)(q21;q21)-investigated gastric MALT lymphoma, *n*	t(11;18)(q21;q21)-positive gastric MALT lymphoma, *n*	Eradication regimen
Yang *et al*[18]	Asian	52	48	38	NR	NR	7-d-14-d triple therapy or 10-d quadruple therapy
Schmelz *et al*[19]	Western	99	99	66	69	7	7-d triple therapy
Sugizaki *et al*[20]	Asian	97	86	84	73	1	7-d triple therapy
Song *et al*[21]	Asian	47	47	35	NR	NR	14-d triple therapy
Li *et al*[22]	Asian	69	69	54	NR	NR	ND-day quadruple therapy
Kim *et al*[24]	Asian	40	35	35	NR	NR	7-d-14-d triple therapy or 7-d-14-d bismuth quadruple therapy
Moleiro *et al*[23]	Western	82	81	77	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
Park *et al*[25]	Asian	87	81	73	NR	NR	7-d-14-d triple therapy
Grgov *et al*[26]	Western	20	20	17	NR	NR	10-d triple therapy
Nonaka *et al*[27]	Asian	12	12	9	NR	NR	7-d triple therapy
Lima *et al*[28]	Western	7	7	5	8	4	7-d triple therapy or 10-d triple therapy
Wündisch *et al*[29]	Western	120	120	96	66	10	14-d dual therapy or 10-d triple therapy
Choi *et al*[30]	Asian	26	26	22	NR	NR	ND-day triple therapy or ND-day bismuth quadruple therapy
Ono *et al*[31]	Asian	13	13	13	NR	NR	7-d-triple therapy
Andriani *et al*[32]	Western	60	53	42	NR	NR	7-d-14-d triple therapy or 10-d bismuth quadruple therapy
Sumida *et al*[33]	Asian	57	57	47	66	7	7-d triple therapy
Stathis *et al*[34]	Western	85	85	66	NR	NR	ND-day triple therapy
Terai *et al*[35]	Asian	70	70	56	22	0	7-d triple therapy
Fischbach *et al*[36]	Western	108	108	35	NR	NR	NR
Kim *et al*[37]	Asian	99	99	84	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
El-Zahabi *et al*[38]	Asian	19	19	8	NR	NR	ND-day quadruple therapy
Hong *et al*[39]	Asian	90	90	85	NR	NR	14-d triple therapy or 14-d bismuth quadruple therapy
Wündisch *et al*[40]	Western	196	193	146	NR	NR	NR

Akamatsu *et al* [41]	Asian	38	38	29	8	6	7-d triple therapy or ND-day quadruple therapy
Wündisch *et al* [42]	Western	120	120	96	65	10	14-d dual therapy or 10-d triple therapy
Montalban *et al* [43]	Western	24	24	22	NR	NR	14-d triple therapy
Chen *et al*[44]	Asian	31	30	24	NR	NR	14-d triple therapy
Taji *et al*[45]	Asian	12	12	7	13	4	14-d triple therapy
Takenaka *et al* [46]	Asian	33	31	26	NR	NR	ND-day triple therapy
Fischbach *et al* [47]	Western	80	80	56	NR	NR	7-d triple therapy
Sheu *et al*[48]	Asian	15	15	11	NR	NR	14-d triple therapy
Caletti *et al*[49]	Western	51	45	25	NR	NR	7-d triple therapy
Nakamura *et al* [50]	Asian	17	17	2	23	7	14-d triple therapy
Liu *et al*[51]	Western	111	111	48	111	44	14-d dual therapy
Bertoni *et al*[52]	Western	62	62	46	NR	NR	7-d triple therapy; 14-d triple therapy or 14-d bismuth quadruple therapy
Ohashi *et al*[53]	Asian	13	13	11	NR	NR	14-d triple therapy
Kim *et al*[54]	Asian	20	20	18	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
Matsushima *et al*[55]	Asian	14	14	10	NR	NR	ND-day triple therapy
Kanda *et al*[56]	Asian	13	12	9	NR	NR	ND-day dual therapy or ND-day triple therapy
Raderer *et al*[57]	Western	22	21	15	NR	NR	ND-day dual therapy or ND-day triple therapy
Nakamura *et al* [58]	Asian	41	41	29	NR	NR	ND-day triple or ND-day quadruple therapy
Ruskoné-Fourmestraux *et al*[59]	Western	34	34	19	NR	NR	14-d triple therapy
Thiede *et al*[60]	Western	97	97	77	NR	NR	14-d dual therapy or 7-d triple therapy
de Jong *et al*[61]	Western	23	23	13	NR	NR	ND-day dual therapy; ND-day triple therapy or ND-day quadruple therapy
Urakami *et al* [62]	Asian	47	44	42	NR	NR	7-d-14-d triple therapy
Papa *et al*[63]	Western	7	7	7	NR	NR	7-d triple therapy
Yamashita *et al* [64]	Asian	21	21	14	NR	NR	14-d triple therapy
Ohashi *et al*[65]	Asian	11	11	9	NR	NR	14-d triple therapy
Nakamura *et al* [66]	Asian	26	25	13	NR	NR	14-d dual therapy; 7-d triple therapy (14-d PPI); 14-d triple therapy
Savio *et al*[67]	Western	76	76	71	NR	NR	NR

Weston *et al*[68]	Western	65	58	38	NR	NR	ND-day triple or ND-day quadruple therapy
Steinbach *et al*[69]	Western	28	28	14	NR	NR	21-d bismuth quadruple therapy
Nobre-Leitão *et al*[70]	Western	17	17	17	NR	NR	14-d triple therapy
Thiede *et al*[71]	Western	84	79	68	NR	NR	NR-day-dual or 7-d-triple therapy
Sackmann *et al*[72]	Western	22	22	12	NR	NR	14-d-dual therapy
Neubauer *et al*[73]	Western	50	50	40	NR	NR	14-d-dual therapy or 7-d-triple therapy
Pinotti *et al*[74]	Western	45	44	30	NR	NR	14-d-triple or quadruple therapy
Savio *et al*[75]	Western	13	12	11	NR	NR	NR-day-triple or quadruple therapy
Bayerdörffer *et al*[76]	Western	33	33	23	NR	NR	14-d-dual therapy
Roggero *et al*[77]	Western	26	25	5	NR	NR	14-d-triple therapy
Wotherspoon *et al*[78]	Western	6	6	5	NR	NR	NR-day-dual or triple therapy

CR: Complete remission rate; *H. pylori*: *Helicobacter pylori*; MALT: Mucosa-associated lymphoid tissue; NR: Not reported; PPI: Proton pump inhibitor.

P-MA of the CR

The overall CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). P-MA highlighted substantial heterogeneity in CR rate reported across studies (I^2 = 92%; P < 0.01) (Figure 3A).

Exploring heterogeneity - subgroup and meta-regression analysis

Considering the high heterogeneity across studies (I^2 = 92%; P < 0.01), a subgroup analysis by study design was conducted. The subgroup analysis revealed that retrospective and prospective studies presented similar overall CR rate after eradication therapy: 75.51% (95%CI: 64.96%-86.07%; I^2 = 96%; P < 0.01) and 75.08% (95%CI: 69.80-80.36; I^2 = 89%; P < 0.01), respectively (Figure 3B). The meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and study risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML and high risk of bias showed the pooled estimate of the CR rate decreased to 0.40 (95%CI: -0.59 to -0.22; P < 0.0001) and 0.43 (95%CI: -0.77 to -0.09; P = 0.0139), respectively. There was no significant difference in outcomes with respect to geographic region (Table 3).

DISCUSSION

GML is rare and typically comprises a low-grade neoplasm[18]. *H. pylori* infection is predominant pathogenic mechanism underlying the development of GML[19], and international guidelines strongly recommend *H. pylori* eradication therapy for all patients irrespective of stage. In localized *H. pylori*-positive GML, bacterial eradication is the preferred initial treatment[79,80].

This study aimed to provide an up-to-date, comprehensive synthesis of evidence regarding *H. pylori* eradication as the sole initial therapy for early-stage GML. We identified prospective and retrospective uncontrolled, single-arm observational studies with a total of 3315 patients with early-stage GML, of which 3003 were *H. pylori*-positive. A total of 2936 patients in whom *H. pylori* was successfully eradicated were included in the analysis. The unavailability of robust comparative studies (*e.g.*, prospective cohort studies) precluded pairwise meta-analysis (PW-MA); instead, a P-MA was conducted. In contrast to comparative PW-MA, which calculates a pooled estimate of effect over 2 groups, P-MA enables the calculation of a grouped overall proportion[81,82]. Though single-group analysis may not produce measures of relative association, it can be useful for estimating the impact of a treatment on a given condition in the absence of higher-quality evidence. This represents an alternative for informed decision making, especially in our field where robust comparative studies are scarce.

Table 3 Meta-regression according to selected covariates

Subgroup	Studies, *n*	Estimate	95%CI	*P* value	I^2, %
Year					
≤ 2015	54	-	-	-	92.5
> 2015	7	0.11	-0.03 to 0.25	0.1188	
Region					92.8
Asian	29	-	-	-	
Western	32	-0.06	-0.15 to 0.03	0.2145	
Proportion of patients with t(11;18)(q21;q21)-positive gastric MALT lymphoma					
≤ 30%	7				
> 30%	4	-0.40	-0.59 to -0.22	< 0.0001	88.6
Risk of bias					92.3
Low	39	-	-	-	
Moderate	21	0.02	-0.07 to 0.12	0.6190	
High	1	-0.43	-0.77 to -0.09	0.0139	

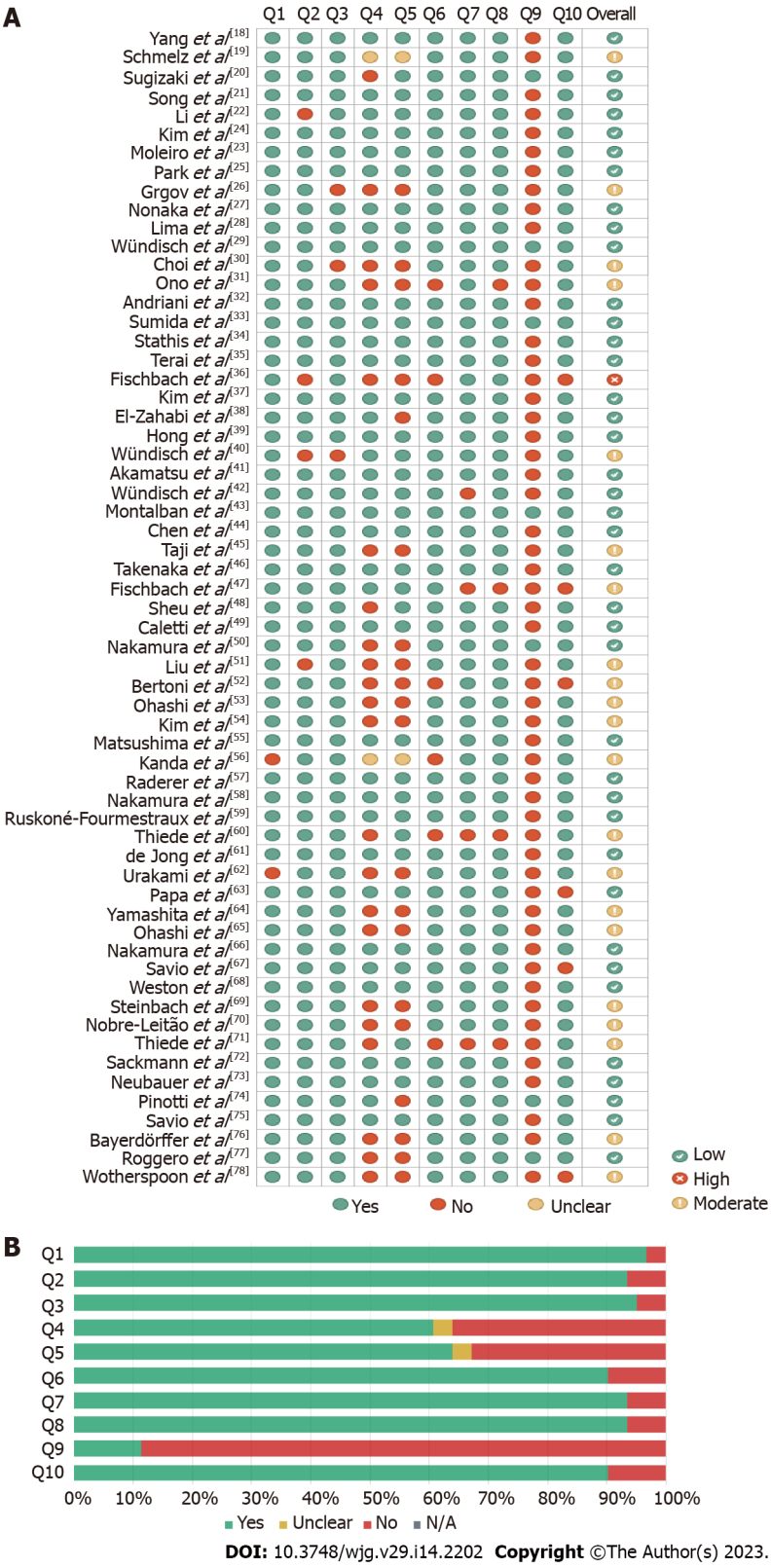
MALT: Mucosa-associated lymphoid tissue.

P-MA highlighted that the overall CR rate of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%), suggesting that *H. pylori* eradication as the sole initial therapy for early-stage GML is effective. These results are similar to those found in a pooled data analysis published in 2010 by Zullo *et al*[12] [77.5% (95%CI: 75.3%-79.7%)]. On the other hand, the substantial heterogeneity observed across studies (I^2 = 92%; P < 0.01) limits, though does not preclude, the interpretation of the pooled overall CR rate. Subgroup analysis revealed that retrospective and prospective studies estimated similar overall CR rates after eradication therapy [75.51% (95%CI: 64.96%-86.07%; I^2 = 96%; P < 0.01) and 75.08% (95%CI: 69.80%-80.36%; I^2 = 89%; P < 0.01), respectively]. Nevertheless, meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and the studies' risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML or high risk of bias decrease in 0.40 (95%CI: -0.59 to -0.22; P < 0.0001) and 0.43 (95%CI: -0.77 to -0.09; P = 0.0139) the pooled estimate of the CR rate, respectively. In this sense, we reiterate the results of Zullo *et al*[12] which highlight the presence of the t(11;18)(q21; q21) translocation as a predictor of lymphoma remission after bacterial eradication. In contrast to the previous pooled analysis[12], our study did not observe significant differences in lymphoma remission between Western and Asian countries.

Hence, our results reaffirm that *H. pylori* eradication should be given as the first-line treatment for localized low-grade GML[8]. The anti-*H. pylori* regimen should be chosen based on regional microbial susceptibility; in many regions, BQT or high-dose PPI clarithromycin-containing triple therapy may be recommended as first-line empirical treatment[83]. In case of eradication failure, second-line treatment should be attempted following the currently recommended algorithm for empirical *H. pylori* eradication or as guided by individual antibiotic susceptibility testing. For patients with GML refractory to *H. pylori* eradication, irradiation and systemic oncological therapies should be used, depending on the stage of the disease. Radiotherapy (RT) is the first-line choice for the treatment of localized GML. Chemotherapy, immunotherapy, or combination chemoimmunotherapy are mainly considered if RT is not feasible or otherwise not indicated[84,85].

To our knowledge, our study is the first systematic review with meta-analysis to assess the CR rate of *H. pylori*-positive early-stage GML after *H. pylori* eradication. Our work has strengths in its design and execution, such as the use of random-effects meta-analysis to address heterogeneity between included studies, subgroup analyses by study design, and meta-regression to explore possible sources of heterogeneity. Nonetheless, the present analysis has several limitations inherent to the included studies and study design. Due to the unavailability of language resources (*e.g.*, professional translators), we could not include studies in languages other than English. Although limiting study inclusion based on the language of publication is a common practice in systematic reviews, it introduces the risk of ignoring key data, referred to as language bias, which may limit the interpretation of our findings[86].

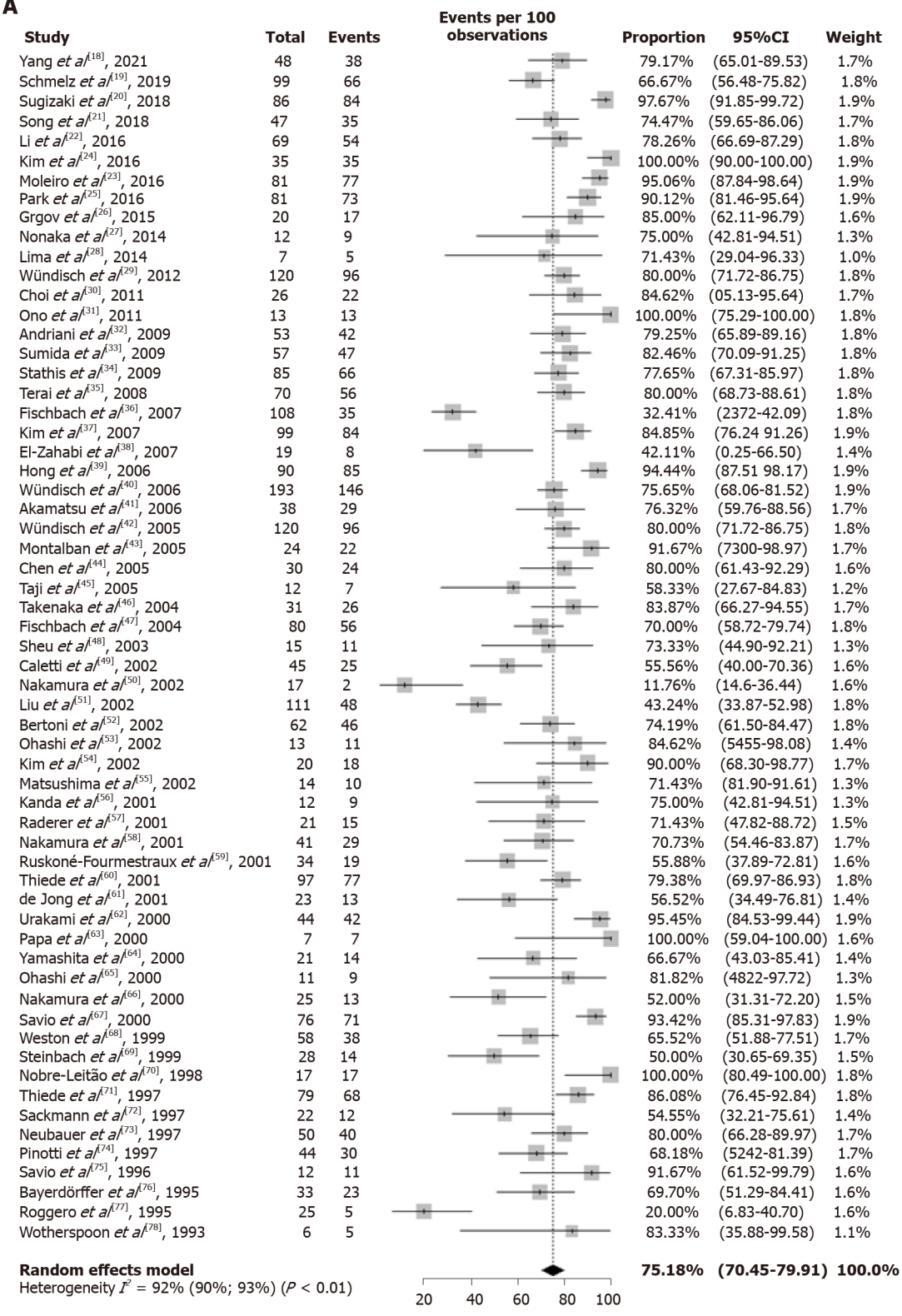
Moreover, discriminated assessments for each JBI Critical Appraisal Tool question across all reports showed that the included series had serious gaps in clinical and demographic information reporting. Thus, exploring possible sources of heterogeneity and identifying predictors of lymphoma remission was difficult. Furthermore, incomplete and non-consecutive inclusion of patients in several studies

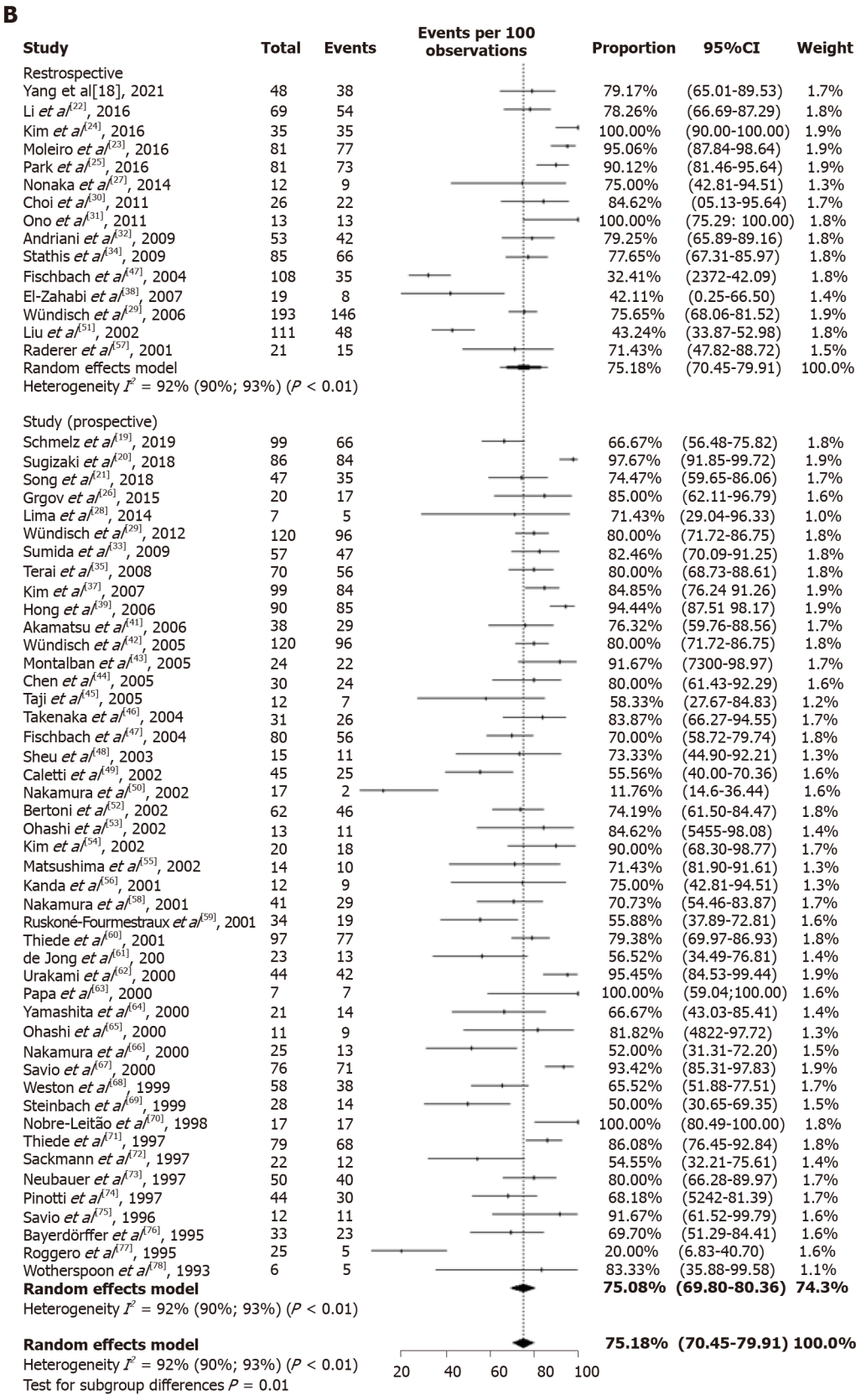


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Figure 2 Risk of bias assessment by the Joanna Briggs Institute Critical Appraisal Tool. The critical appraisal checklist for case series consists of 10 questions: Q1: Were there clear criteria for inclusion in the case series? Q2: Was the condition measured in a standard, reliable way for all participants included in the case series? Q3: Were valid methods used for identification of the condition for all participants included in the case series? Q4: Did the case series have consecutive inclusion of participants? Q5: Did the case series have complete inclusion of participants? Q6: Was there clear reporting of the demographics of the participants in the study? Q7: Was there clear reporting of clinical information of the participants? Q8: Were the outcomes or follow-up results of cases clearly reported? Q9: Was there clear reporting of the presenting site(s)/clinic(s) demographic information? Q10: Was statistical analysis appropriate? The percentage of risk of bias was calculated by the number of "yes" answers selected in the checklist. Questions with "not applicable" answers were not considered in the calculation. The risk of bias was classified using the following categories: High (scores up to 49.0%), moderate (scores between 50.0% and 70.0%), and low (scores above 70.0%). A: Overall risk of bias; B: Risk of bias summary: Discriminated assessments for each question across all studies. N/A: Not applicable.

A


Study
Total
Events
Events per 100 observations
Proportion
95%CI
Weight
Yang et al[18], 2021 48 38 79.17% (65.01-89.53) 1.7%
Schmelz et al[19], 2019 99 66 66.67% (56.48-75.82) 1.8%
Sugizaki et al[20], 2018 86 84 97.67% (91.85-99.72) 1.9%
Song et al[21], 2018 47 35 74.47% (59.65-86.06) 1.7%
Li et al[22], 2016 69 54 78.26% (66.69-87.29) 1.8%
Kim et al[24], 2016 35 35 100.00% (90.00-100.00) 1.9%
Moleiro et al[23], 2016 81 77 95.06% (87.84-98.64) 1.9%
Park et al[25], 2016 81 73 90.12% (81.46-95.64) 1.9%
Grgov et al[26], 2015 20 17 85.00% (62.11-96.79) 1.6%
Nonaka et al[27], 2014 12 9 75.00% (42.81-94.51) 1.3%
Lima et al[28], 2014 7 5 71.43% (29.04-96.33) 1.0%
Wündisch et al[29], 2012 120 96 80.00% (71.72-86.75) 1.8%
Choi et al[30], 2011 26 22 84.62% (05.13-95.64) 1.7%
Ono et al[31], 2011 13 13 100.00% (75.29-100.00) 1.8%
Andriani et al[32], 2009 53 42 79.25% (65.89-89.16) 1.8%
Sumida et al[33], 2009 57 47 82.46% (70.09-91.25) 1.8%
Stathis et al[34], 2009 85 66 77.65% (67.31-85.97) 1.8%
Terai et al[35], 2008 70 56 80.00% (68.73-88.61) 1.8%
Fischbach et al[36], 2007 108 35 32.41% (2372-42.09) 1.8%
Kim et al[37], 2007 99 84 84.85% (76.24 91.26) 1.9%
El-Zahabi et al[38], 2007 19 8 42.11% (0.25-66.50) 1.4%
Hong et al[39], 2006 90 85 94.44% (87.51 98.17) 1.9%
Wündisch et al[40], 2006 193 146 75.65% (68.06-81.52) 1.9%
Akamatsu et al[41], 2006 38 29 76.32% (59.76-88.56) 1.7%
Wündisch et al[42], 2005 120 96 80.00% (71.72-86.75) 1.8%
Montalban et al[43], 2005 24 22 91.67% (7300-98.97) 1.7%
Chen et al[44], 2005 30 24 80.00% (61.43-92.29) 1.6%
Taji et al[45], 2005 12 7 58.33% (27.67-84.83) 1.2%
Takenaka et al[46], 2004 31 26 83.87% (66.27-94.55) 1.7%
Fischbach et al[47], 2004 80 56 70.00% (58.72-79.74) 1.8%
Sheu et al[48], 2003 15 11 73.33% (44.90-92.21) 1.3%
Caletti et al[49], 2002 45 25 55.56% (40.00-70.36) 1.6%
Nakamura et al[50], 2002 17 2 11.76% (14.6-36.44) 1.6%
Liu et al[51], 2002 111 48 43.24% (33.87-52.98) 1.8%
Bertoni et al[52], 2002 62 46 74.19% (61.50-84.47) 1.8%
Ohashi et al[53], 2002 13 11 84.62% (5455-98.08) 1.4%
Kim et al[54], 2002 20 18 90.00% (68.30-98.77) 1.7%
Matsushima et al[55], 2002 14 10 71.43% (81.90-91.61) 1.3%
Kanda et al[56], 2001 12 9 75.00% (42.81-94.51) 1.3%
Raderer et al[57], 2001 21 15 71.43% (47.82-88.72) 1.5%
Nakamura et al[58], 2001 41 29 70.73% (54.46-83.87) 1.7%
Ruskoné-Fourmestraux et al[59], 2001 34 19 55.88% (37.89-72.81) 1.6%
Thiede et al[60], 2001 97 77 79.38% (69.97-86.93) 1.8%
de Jong et al[61], 2001 23 13 56.52% (34.49-76.81) 1.4%
Urakami et al[62], 2000 44 42 95.45% (84.53-99.44) 1.9%
Papa et al[63], 2000 7 7 100.00% (59.04-100.00) 1.6%
Yamashita et al[64], 2000 21 14 66.67% (43.03-85.41) 1.4%
Ohashi et al[65], 2000 11 9 81.82% (4822-97.72) 1.3%
Nakamura et al[66], 2000 25 13 52.00% (31.31-72.20) 1.5%
Savio et al[67], 2000 76 71 93.42% (85.31-97.83) 1.9%
Weston et al[68], 1999 58 38 65.52% (51.88-77.51) 1.7%
Steinbach et al[69], 1999 28 14 50.00% (30.65-69.35) 1.5%
Nobre-Leitão et al[70], 1998 17 17 100.00% (80.49-100.00) 1.8%
Thiede et al[71], 1997 79 68 86.08% (76.45-92.84) 1.8%
Sackmann et al[72], 1997 22 12 54.55% (32.21-75.61) 1.4%
Neubauer et al[73], 1997 50 40 80.00% (66.28-89.97) 1.7%
Pinotti et al[74], 1997 44 30 68.18% (5242-81.39) 1.7%
Savio et al[75], 1996 12 11 91.67% (61.52-99.79) 1.6%
Bayerdörffer et al[76], 1995 33 23 69.70% (51.29-84.41) 1.6%
Roggero et al[77], 1995 25 5 20.00% (6.83-40.70) 1.6%
Wotherspoon et al[78], 1993 6 5 83.33% (35.88-99.58) 1.1%
Random effects model 75.18% (70.45-79.91) 100.0%
Heterogeneity I^2 = 92% (90%; 93%) (P < 0.01)
20
40
60
80
100



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Figure 3 Overall complete remission rate of *Helicobacter pylori*-positive early-stage gastric mucosa-associated lymphoid tissue lymphoma. A: After eradication therapy; B: After eradication therapy by study design. CI: Confidence interval.

compromises the reliability of their results and increases the risk of bias. Another limitation was the failure to report the confirmation method for *H. pylori* eradication, which could be a covariate explaining the heterogeneity between studies. Inadequate reporting was an important reason for the exclusion of

studies during screening and a complicating factor for data extraction. Observational studies evaluating the CR of GML after bacterial eradication should stratify the observed outcome according to *H. pylori* infection status. Furthermore, it is necessary to discriminate the lymphoma stage in *H. pylori*-positive patients undergoing treatment. In fields in which reliable and robust studies are scarce, proper reporting of the available evidence is vital to inform clinical practice. Therefore, this meta-analysis should be interpreted in the context of these limitations.

CONCLUSION

This comprehensive evidence synthesis suggests the effectiveness of *H. pylori* eradication as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the interpretation of the pooled overall CR rate, our study is a relevant alternative for informing clinical practice. Further robust comparative observational studies are needed to identify predictive factors for GML remission following *H. pylori* eradication and to provide more reliable evidence in our field.

ARTICLE HIGHLIGHTS

Research background

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade, B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. As such, clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML.

Research motivation

Studies that aim to evaluate the effects of *H. pylori* eradication on early-stage GML are generally small and heterogenous single-arm uncontrolled observational studies. Hence, we recognized the need for an updated powerful statistical synthesis of the available evidence regarding the practical effect of *H. pylori* eradication as sole initial therapy for early-stage GML.

Research objectives

We aimed to perform a systematic review with an up-to-date proportional meta-analysis (P-MA) to assess the complete remission (CR) rate of *H. pylori*-positive early-stage GML after bacterial eradication therapy.

Research methods

We performed independent computer-assisted searches of PubMed/MEDLINE, Embase and Cochrane Central databases culling reports published before September 2022. Prospective and retrospective observational studies evaluating the CR rate of early-stage GML after bacterial eradication therapy in *H. pylori*-positive patients were eligible for inclusion. The risk of bias was assessed using the JBI Critical Appraisal Tools. We followed the random-effects model to calculate the pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI). We used Cochran's Q test and I^2 statistic to assess the heterogeneity and inconsistency, and we set the threshold for heterogeneity as $P < 0.01$ and $I^2 > 50\%$, respectively. Subgroup and meta-regression analyses were conducted to explore potential sources of heterogeneity.

Research results

P-MA highlighted that the overall CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). On the other hand, the substantial heterogeneity observed across studies ($I^2 = 92\%$; $P < 0.01$) limits, but does not preclude, the interpretation of the pooled overall CR rate. Subgroup analysis revealed that retrospective and prospective studies presented similar overall CR rate estimates after eradication therapy: 75.51% (95%CI: 64.96%-86.07%; $I^2 = 96\%$; $P < 0.01$) and 75.08% (95%CI: 69.80%-80.36%; $I^2 = 89\%$; $P < 0.01$), respectively. Nevertheless, meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and the studies' risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML and high risk of bias decrease in 0.40 (95%CI: -0.59 to -0.22; $P < 0.0001$) and 0.43 (95%CI: -0.77 to -0.09; $P = 0.0139$) the pooled estimate of the CR rate, respectively.

Research conclusions

Comprehensive evidence synthesis suggests the effectiveness of *H. pylori* eradication as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the

interpretation of the pooled overall CR rate, the present study is a relevant alternative for informing clinical practice.

Research perspectives

Inadequate reporting was an important reason for the exclusion of studies during screening and a complicating factor for data extraction. As reliable and robust studies are scarce in our field, we emphasize that proper reporting of the available evidence is vital to inform clinical practice. Further robust comparative observational studies are needed to identify predictive factors for GML remission following *H. pylori* eradication and to provide more reliable evidence in our field.

FOOTNOTES

Author contributions: Lemos FFB, Castro CT, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F contributed to the conceptualization of the manuscript; Lemos FFB, Castro CT, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F designed the study methodology; Lemos FFB, Castro CT, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS and Delgado HA were responsible for manuscript visualization; Lemos FFB, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS, Delgado HA, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F contributed to the investigation; Lemos FFB, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS, Delgado HA performed formal analysis; Lemos FFB wrote the original draft; Castro CT and Silva Luz M were responsible for manuscript editing; Castro CT, Silva Luz M, Teixeira KN, Souza CL, and Oliveira MV were responsible for manuscript writing and review; and Freire de Melo F supervised the writing of the original draft.

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REVIEW

Intersections between innate immune response and gastric cancer development

Franz Villarroel-Espindola, Troy Ejsmentewicz, Roxana Gonzalez-Stegmaier, Roddy A Jorquera, Esteban Salinas

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Abstract

Worldwide, gastric cancer (GC) is the fifth most commonly diagnosed malignancy. It has a reduced prevalence but has maintained its poor prognosis being the fourth leading cause of deaths related to cancer. The highest mortality rates occur in Asian and Latin American countries, where cases are usually diagnosed at advanced stages. Overall, GC is viewed as the consequence of a multifactorial process, involving the virulence of the *Helicobacter pylori* (*H. pylori*) strains, as well as some environmental factors, dietary habits, and host intrinsic factors. The tumor microenvironment in GC appears to be chronically inflamed which promotes tumor progression and reduces the therapeutic opportunities. It has been suggested that inflammation assessment needs to be measured qualitatively and quantitatively, considering cell-infiltration types, availability of receptors to detect damage and pathogens, and presence or absence of aggressive *H. pylori* strains. Gastrointestinal epithelial cells express several Toll-like receptors and determine the first defensive line against pathogens, and have been also described as mediators of tumorigenesis. However, other molecules, such as cytokines related to inflammation and innate immunity, including immune checkpoint molecules, interferon-gamma pathway and NETosis have been associated with an increased risk of GC. Therefore, this review will explore innate immune activation in the context of premalignant lesions of the gastric epithelium and established gastric tumors.

Key Words: Gastric cancer; Toll-like receptor; *Helicobacter pylori*; Nuclear factor kappa B; Neutrophils

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Core Tip: Premalignant cascade of gastric cancer starts with a chronic gastritis, and evolves to atrophic gastritis, intestinal metaplasia, dysplasia and finally the carcinoma. During the process, different immune responses contribute to inflammation of the gastric epithelium. Our work compiles studies related to the innate immune response with a focus on molecular and cellular features such as, Toll-like receptors, neutrophils, cytokines and socioeconomic factors, as crucial players during the precancerous cascade and the cancer onset.

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INTRODUCTION

Globally, gastric cancer (GC) is the fifth most commonly diagnosed malignancy. Although there has been a reduction in its incidence, its poor prognosis makes it the fourth leading cause of cancer related deaths per year[1], and around 86% of all GC cases in 2018 occurred in countries with a high or very high Human Development Index, where 60% of the total cases occurred in Eastern and South-Eastern Asia[2]. GC development is a multistep process initiated by the transition of normal mucosa to non-atrophic gastritis. This superficial gastritis may progress to atrophic gastritis, then intestinal metaplasia and finally to dysplasia and adenocarcinoma[3]. Overall, GC is viewed as the consequence of a multifactorial process involving environmental factors (socioeconomic status, smoking and alcohol consumption), dietary habits (diets rich in salt and poor in antioxidants) and intrinsic factors (ethnicity, genetic background, age and sex)[4,5]. Recently, a meta-analysis and prospective cohort study demonstrated in the Chinese population, that healthy lifestyle factors such as abstention from smoking, non-consumption of alcohol, low consumption of preserved foods, and frequent intake of fresh fruits and vegetables and all of these factors in combination can significantly reduce the relative and absolute risk of incidence of GC. Although, the individual carries a high polygenic risk of GC based on the presence of 112 single-nucleotide polymorphisms[6]. This observation suggests that some intrinsic host factors, like the genetic background, may be secondary to external or environmental aspects during GC onset.

Most of the malignant gastric tumors correspond to the histological type of adenocarcinoma (approximately 90%), with a lower percentage of lymphomas of the mucosa-associated lymphoid tissue, leiomyosarcomas and other rarer tumors[7,8]. The adenocarcinomas have been divided classically into two histological subtypes: Diffuse and intestinal, each of which have differences in their presentations depending on the anatomic subsite, age when diagnosed, sex, race, demographical distribution and socio-economic situation[7-9]. More recent molecular and genomic classifications have defined four major genomic subtypes of GC: The Epstein-Barr virus (EBV) infected tumors; genomically stable tumors; chromosomally unstable tumors; and tumors with microsatellite instability (MSI)[5], all of which offer a poor prognosis and different molecular profiles.

It is very well documented that GC is strongly associated with infectious agents such as the bacterium *Helicobacter pylori* (*H. pylori*) and, recently, the EBV[5]. Approximately 15%–20% of human cancers are provoked by cancer-causing viruses[10]; however, the specific role of EBV in GC development is not clear as of yet. Although the World Health Organization has categorized *H. pylori* as a group 1 carcinogen[11], the role of other bacteria in causing cancer is controversial; studies have shown that some bacteria, such as *Fusobacterium nucleatum*[12], and *Porphyromonas spp.*[13,14] play a role in the development of colon, oral and other digestive cancers. Nevertheless, all those microorganisms can promote a local inflammatory status and a parallel activation of protumoral pathways.

Innate immunity represents the first barrier against pathogens, and epithelial cells of the gastric mucosa are the first line of immunity against, for example, an *H. pylori* infection. In response to an infection, many physiological adaptations are observed, such as an increase in vascular diameter and permeability along with an overexpression of cell-adhesion molecules on endothelial cells which promotes the extravasation of myeloid cells into the inflamed site of infection. The characteristics of an inflamed microenvironment are low levels of glucose and a scarcity of oxygen due to an altered metabolism, increased oxygen consumption by neutrophils, and a reduced oxygen supply due to disrupted perfusion[15]. It is within such a hypoxic microenvironment that immune cells kill and prevent the spread of invading microorganisms. Accumulating evidence suggests that chronic inflammation, either non-infectious such as in autoimmune disorders or, as a result of a pathogen infection, is connected to cancer development. At the same time, the crosstalk between innate and adaptive immunity is critical for the successful eradication of different pathogens and tumor cells.

The aim of this review is to provide an overview of innate immune activation in the context of gastrointestinal malignancies, focusing on the premalignant lesions of the epithelium and the gastric tumor microenvironment. During the transition from atrophic gastritis to the final carcinoma, some microorganisms will play a determinant role promoting the neoplastic transformation or contributing with a particular tumor phenotype. This work compiles studies related to Toll-like receptors (TLRs), neutrophils, cytokines and pathogens, as crucial players during the precancerous cascade and the cancer onset (Figure 1), allowing the correlation of those aspects with clinical and socioeconomic variables.

STARTERS AND MEDIATORS OF THE INNATE IMMUNE RESPONSE IN GASTRIC TISSUE

Gastro-intestinal epithelial cells express several TLRs that can respond to exogenous infectious ligands or pathogen-associated molecular patterns (PAMPs). TLRs are the most important class of pattern recognition receptors (PRRs). These transmembrane proteins present a distinctive Leucine-Rich Repeat extracellular domain that confers specificity to their ligands[16], and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the toll/IL-1R homology domain[17]. Up to now, the TLR family consists of ten (TLR1-TLR10) and twelve (TLR1-TLR9 and TLR11-TLR13) members identified in humans and mice respectively[18]. These receptors are expressed in various immune cells, including macrophages, Dendritic cells (DCs), B cells, specific types of T cells, and even in non-immune cells such as fibroblasts and epithelial cells; their activation leads to the induction of inflammatory cytokines, chemokines, antigen-presenting molecules, and costimulatory molecules[15,17,19].

Epithelial cells from the gastric mucosa are considered as the first line of innate immunity against gastrointestinal pathogens, including *H. pylori* infection, and the PRRs have shown a wide range of expression in normal and pathological tissue (Table 1).

Human gastric epithelial cells and tumor cells were found to express both TLR2 and TLR4 and both receptors are described as responsible for the *H. pylori* lipopolysaccharides (LPS) recognition. However, the results are contradictory and have not accurately probed the role of those receptors due both to the diversity of the host's immune system and the pathogenicity of the *H. pylori* strain[20,21]. TLR2 is the most extensively expressed gene among all the TLRs in gastric tumors and high levels of TLR4 are associated with a higher risk of GC[22-24].

On the other hand, TLR3 and TLR4 have been implicated in several disorders related to the gastroesophageal reflux disease spectrum and largely documented, including the expression of both receptors and expression of their downstream products, such as cyclooxygenase-2, IL-8, nuclear factor-κB (NF-κB), and nitric oxide in human tissue samples and *ex vivo* cell cultures from the esophagus, the esophageal-gastric junction and the stomach[25].

TLR5 is expressed within the esophageal epithelium and has been shown to increase in a stepwise manner with progression from normal to dysplastic and eventually neoplastic states[26]. In addition, it is well documented that TLR5 is present in both primary gastric epithelial cells and gastric tumor cell lines[22,24,27]; however, the role of TLR5 during the gastric precancerous cascade is not yet clear.

TLR5 is responsible for flagellin recognition. *H. pylori* flagellin seems to be a less potent stimulator compared with other flagellins[28] but has a significant role in long-term bacterial persistence. The lack of TLR5 activity in response to *H. pylori* flagellin is caused mainly by the amino acid residues variation R89, L93, and E114 described as hotspots for binding TLR5 which, replaced with threonine (R89T), lysine (L93K), and aspartate (E114D) in *H. pylori* flagellin, lead to receptor evasion[29]. TLR5 instead recognizes CagL and CagY, two proteins from the type IV secretion system (T4SS) of *H. pylori*, and both have immunoregulatory properties[30,31]. A high TLR5 expression has been suggested to have a better prognosis amongst young GC patients in an early stage of disease, and this better outcome may be associated with a non-distant metastasis and an intestinal-type cancer[32].

TLR9, the only TLR with both anti- and pro-inflammatory roles, is involved in the recognition of *H. pylori* DNA, and the promotion or suppression role of TLR9 will depend on the gastric environment [22]. TLR9 expression has been shown to be up-regulated in *H. pylori* infected gastric tissue compared with non-infected tissue, and it was not related to the presence of tumor cells, suggesting that increased TLR9 expression was specifically associated with *H. pylori* infection[33]. It is reported that TLR9 interaction with *H. pylori* and *H. pylori* DNA, triggers an IL-8 secretion response mediated by the NF-κB pathway[34].

The role of TLR9 in cancer is not absolutely clear, but patients with stage II of GC and a high TLR9 expression had a better prognosis than cases with lower levels[32].

Other TLRs have been described, TLR1, TLR7, TLR8, and TLR10, but further studies are required in order to understand their role in GC and in *H. pylori* infected individuals, as well as other pathogens[22,23,35]. However, high levels of TLR10 expression have been observed in gastric biopsy samples from subjects with *H. pylori* and, when NCI-N87 gastric cells were co-cultured with the bacteria, both TLR10 and TLR2 mRNA levels were upregulated[35]. Those results suggest that TLR10 is a functional receptor and that TLR2/TLR10 heterodimer functions in *H. pylori* LPS recognition[35].

Table 1 Expression of Toll-like receptors in esophageal and gastric epitheliums

PRR	Organ	Model	Method	Ligand	Observation	Reference
TLR1/2	Esophagus	Esophageal carcinoma and premalignant lesions	IHQ	Triacyl lipopeptide	Receptor upregulation in tumor and dysplasia	[22,171]
TLR2	Stomach; esophagus	Human ADC and premalignant lesions; *H. pylori* infection mice model and*in vitro* culture	IHQ; RT-qPCR	Microbial lipopeptide	Receptor upregulation in tumor cores. Increased tumorigenesis; constitutive expression in TE-1 cell line	[21,22,27,32,172-174]
TLR3	Stomach; esophagus	Human gastric and esophageal carcinoma	IHQ; RT-qPCR	dsRNA	Increased receptor levels correlate with poor prognosis; increased expression on EAC-derived cell lines	[25,174-176]
TLR4	Stomach; esophagus	Human ADC and premalignant lesions; *H. pylori* infection; esophageal carcinoma	IHQ; RT-qPCR	LPS	Upregulation in tumor cores; weak association with clinicopathologic variables; high expression correlates with poor prognosis; upregulation of IL-8 and COX-2 in BE	[21,24,25,27,32,171,173-179,182]
TLR5	Stomach; esophagus	Human ADC and premalignant lesions; *H pylori* infection	IHQ	Flagellin	Highly expressed; upregulation in tumor and older patients; association with necrosis and tumor growth in the stomach; overexpression in dysplastic lesions of BE; no association with EAC prognosis	[26,27,32,177]
TLR6	Esophagus	Esophageal carcinoma and human dysplasia	IHQ; IF	Diacyl lipopeptide	Upregulated in tumor tissue	[22,171]
TLR7	Stomach; esophagus	Human ADC and normal tissue	IHQ; WB; RT-qPCR	ssRNA	Downregulated in gastric tumors; high levels correlate with a better outcome in GC; constitutive expression in TE-1 cell line; association between expression and tumor grade in ESCC	[22,32,173,174,176,180,183]
TLR9	Stomach; esophagus	Human ADC	IHQ; RT-qPCR	ssDNA; dsDNA	Upregulated in early tumors; correlation with better prognosis in GC; association with histopathological grade in ESCC and dysplasia; high expression in EAC correlates with advanced tumor stage and metastasis	[24,25,32-34,173,175,176,181,184]
TLR10	Stomach	Human biopsy	RT-qPCR	ssDNA; dsDNA	Upregulated by *H. pylori*	[35]

ADC: Adenocarcinoma; BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; IF: Immunofluorescence; IHQ: Immunohistochemistry; PRR: Pattern recognition receptor; RT-qPCR: Reverse transcription and quantitative PCR; TE-1: Human cell line derived from esophageal cancer; TLR: Toll-like receptors; WB: Western blot.

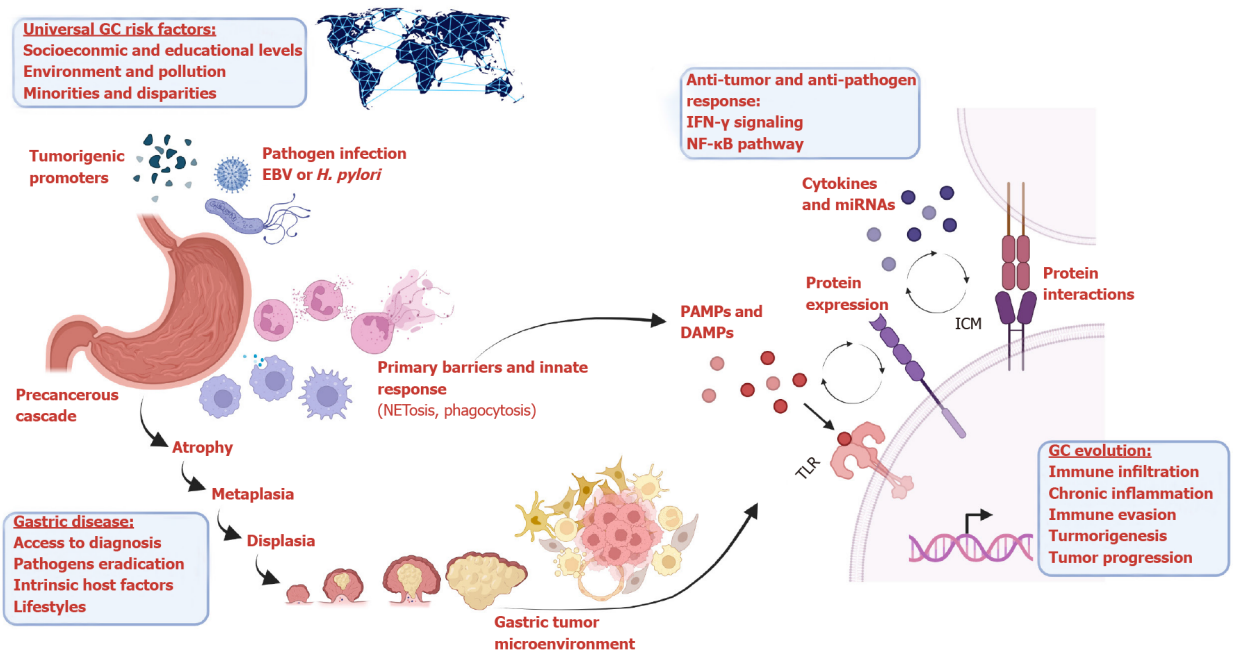
From a cellular perspective, neutrophils are the most abundant white blood cells in human blood and also considered as part of the first line of defense against infections by pathogens[36]. These cells have the ability to capture and destroy invading microorganisms and participate as mediators of inflammation. Phagocytosis and formation of neutrophil extracellular traps are part of the cellular mechanisms for pathogen elimination, as well as granules releasing[36-38].

Neutrophils extracellular traps (NETs) formation, known as NETosis is a process of releasing extracellular web-like structures, and is described as a coat consisting of decondensed chromatin filaments, histones and antimicrobial proteins[39]. NETosis is a mechanism of innate immunity to contain and prevent microbial spread, and eliminate bacteria[40].

H. pylori can activate different cells of innate immunity, including neutrophils, and these activated cells recognize *H. pylori* infection through different receptors, such as TLR2, TLR4, and TLR9[34,41,42]. TLR5 has not been detected in neutrophils localized in the lamina propria during *H. pylori* gastritis[24]. The activation and recruitment of neutrophils is stimulated by *H. pylori* neutrophil-activating protein, or HP-NAP[37,43]. TLR2 interacts with HP-NAP for the secretion of IL-8[41]. However, the interaction between neutrophils and *H. pylori* appears to be complex and contradictory and shows the development of different mechanisms of immune evasion, including NET degradation, the increase in bacterial resistance mediated by the modification of proteins or surface polysaccharides, or the suppression of NET formation[44].

H. pylori has shown a selective alteration of neutrophils function mediated by the inactivation of NADPH Oxidase and superoxide release[45]. In addition, the bacterium performs lipid A modification mediated by lipid A phosphatases to resist the polymyxin, an antimicrobial peptide[46]. Another study remarked on the presence of an outer membrane-associated nuclease that can degrade extracellular DNA, where the ability to degrade exogenous DNA was originally proposed as a purine source uptake mechanism[47], but it could also have the potential role of degrading NETs[48].

Although NETosis was described as an antimicrobial process, it has been described in other pathologies, including cancer. The first study that provided evidence on NET in cancer was Berger-Achituv *et al*[49] studying the Ewing sarcoma. The authors proposed NET as a pro-tumor effect and the



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Figure 1 Graphical abstract. Intersections between innate immune response and gastric cancer (GC) development is driven by common mediators, such as molecular pathways [nuclear factor-kappa B (NF-κB) and interferon-gamma (IFN-γ)], cellular processes (neutrophil and myeloid cells activation), and activators/inducers [pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), tumor-antigens]. However, universal risk factors are identified affecting globally to any human being, which depending on extrinsic and intrinsic host factors might facilitate the progression of the precancerous cascade to GC. This image is created in BioRender.com. EBV: Epstein-Barr virus; *H. pylori*: *Helicobacter pylori*; ICM: Immune checkpoint molecule; TLR: Toll-like receptor.

possibility of using this parameter as a poor prognostic biomarker[49].

Regarding GC, the first study was reported by Yang *et al*[50]. The authors found the correlation of NET formation with TNM status and a significant increase in the formation of fibrin and thrombin, however, the focus was on peripheral circulation. More recently, the formation of NETs within the gastric tumor microenvironment including immunofluorescent staining of Neutrophil Elastase (NE) and citrullinated-histone 3[51,52] showed that NETs are more abundant in the tumor core than in the adjacent non-tumor tissue[51,52], and the plasma from GC patients revealed the capacity of NET formation *in vitro*[52]. NETs measured in peripheral blood have been shown to be significantly correlated with GC and staging, and its levels decrease after surgery[50-52].

A very recent report showed that abdominal infectious complications after gastrectomy would stimulate neutrophils to release NETs both in peripheral blood and the abdominal cavity, facilitating GC metastasis *in vitro* and *in vivo* dependent on transforming growth factor (TGF)-β signaling[53]. The formation of NETs has an important role in the epithelial-mesenchymal transition and gastric tumor progression, because NETosis may induce proliferation, invasion, migration, and a mesenchymal phenotype, in addition to its immune role, which makes it difficult to be therapeutically targeted.

ACCOMPLICE CELLS AND MEDIATORS OF AN ANTI-TUMOR RESPONSE

Additionally, the macrophages have been demonstrated to be important cells for the innate immune system in healthy and tumor tissue. Within the tumor microenvironment (TME), macrophages are known as tumor-associated macrophages (TAMs) and play a key role in the recognition and clearance of foreign and damaged cells, as well as in tumor development and the response to several cancer therapies.

Macrophages can infiltrate solid tumors modulating T cell activity within the TME, and often undergo phenotype polarization in response to stimuli or inhibitory factors, either to pro-inflammatory (M1) or anti-inflammatory (M2) subtypes, which cause immune response or immune escape of the tumors respectively[54-57]. The general consensus is that TAMs are usually pro-tumorigenic. These cells are recruited by tumor-derived chemokines and produce low levels of inflammatory cytokines, promote Th2-T cell response, favor wound healing, and increase angiogenesis and metastases[55,56].

IL-6 and tumor necrosis factor (TNF)-α are both pro-inflammatory cytokines, exerting pro-tumoral functions, including the promotion of angiogenesis and metastasis, and these molecules can be secreted by myeloid cells and leukocytes under different conditions and stimuli[54-56]. In parallel to classic

immunomodulators such as cytokines and chemokines, some microRNAs (miR) have shown a significant impact on macrophages activity[57]. miR-125b, miR-127, miR-155, miR-181 and miR-451 are significantly upregulated in M1 macrophages, whereas miR-125a-5p, miR-146a, miR-145-5p, miR-143-3p are highly expressed in M2 macrophages[58,59].

miR-155 directly targets the expression of the IL-13 receptor α1, thereby inhibiting STAT6 activation and promoting M1 polarization[59]. miR-155 knockdown in myeloid cells induces faster tumor growth, reduction of M1-macrophages and enrichment of pro-tumor cytokines within the TME[60]. In addition, miR-125b overexpression enhanced responsiveness to interferon (IFN)-γ, through the targeting of IRF4 and increased expression of pro-inflammatory cytokines[58,61].

miR-187, miR-146a, let-7e, and miR-92a are considered anti-inflammatory miRs because they downregulate IL-6 and TNF-α in human macrophages by targeting the TLRs signaling[57,62]. The NF-κB-dependent miR-146a expression is induced in monocytes and macrophages upon triggering of TLR4 to act as a modulator of the inflammatory response[61]. miR-155 is key to modulating genes related to M2/pro-Th2 phenotype in macrophages, and includes CCL18, SERPINE, CD23 and DC-SIGN[56]. In addition, other microRNAs will modulate directly or indirectly the NF-κB or TLR activity, such as miR9, miR-21, miR-29b, and those can be expressed and released by both TAMs and solid tumors in exosomes [57,62-65]. Due to the inflammatory microenvironment and oncogenic mutations, a significant number of human cancers have constitutive miRs deregulation affecting NF-κB activity, cytokine production and hallmarks of cancer such as apoptosis, proliferation and tumor survival[57,62,65].

Previous studies demonstrated that Gastric Epithelial Cells (GECs) function as antigen presenting cells by constitutively expressing MHC class II[66]. Interestingly, *H. pylori* infection induces up-regulation of costimulatory molecules (CD86 and CD80) among GECs[66], suggesting its potential to be used as a local bridge between innate and acquired immunity; however, the capacity to play a role secreting cytokines and polarizing macrophages requires further studies.

The inhibition of the polarization of pro-inflammatory macrophages can accelerate the development of precancerous lesions in GC[67]. In addition, when TAMs spread in the peritoneum of GC patients, these cells normally are polarized to an anti-inflammatory subtype (M2), which can promote the growth and progression of GC when the tumor exists[68]. In fact, high densities of TAMs are associated with poor survival in GC patients[68,69].

INTRINSIC AND EXTRINSIC MODULATORS OF INFLAMMATION AND PRECANCEROUS LESIONS

Currently, there is enough evidence based on epidemiological, molecular and pathological studies that persistent infection with *H. pylori* is a risk factor for the development of gastric adenocarcinoma[70-73], estimating an increment in the relative risk by 3–6 times in infected people which might represent over 80% of all distal GC cases and some with proximal gastric tumors[1,74,75]. The prevalence of *H. pylori* infection is extraordinarily high, infecting 50% of the world's population[1,76-78].

H. pylori is a Gram-negative bacterium which colonizes the human stomach and promotes a full immune response locally and sustainably[19,79,80]. Strains of *H. pylori* are grouped into two broad families tentatively named type I and type II, which are based on whether they express or not the vacuolating cytotoxin (VacA) and the CagA antigen (cytotoxin-associated gene A)[81].

One of the major determinants of *H. pylori*'s virulence is the cag pathogenicity island (cag PAI)[82-84]. This cagPAI is a 40-kb DNA region surrounding the *cagA* gene that contains about 27-31 genes that encode a bacterial type IV secretion system acting as a syringe-like structure, which allows for the delivery of bacterial effector molecules into host gastric epithelial cells[85]. During infection with *H. pylori*, CagA is translocated into epithelial cells, and it is tyrosine-phosphorylated in the EPIYA motifs by the proto-oncogene tyrosine-protein kinase Src and the members of the Abelson family of non-receptor tyrosine kinases[86,87], which results in the interaction with various intracellular signaling pathways, triggering changes in the cytoskeleton, in the morphology and in the mobility of the host cells [86,87].

Compared with cagA– strains, *H. pylori* cagA+ strains significantly increase the risk of developing severe gastritis, atrophic gastritis, peptic ulcer disease and distal GC. In fact, people infected with cagA+ strains have higher degrees of gastric inflammation and epithelial cell damage than people from whom cagA- strains have been isolated[88]. People infected with cagA+ *H. pylori* strains have an enhanced expression of IL-1α, IL-1β, and IL-8 in gastric biopsies compared to uninfected persons or patients infected with cagA- strains[89]. Keeping that consideration in mind, proinflammatory cytokines will be up-regulated by a local infection; however, the magnitude of that systemic response and the profile of released cytokines and chemokines will depend also on host factors.

Regarding host factors, many polymorphisms in genes related to inflammation and innate immunity, such as cytokines and MHC molecules, have been reported to be associated with an increased risk of GC [90-92]. Among the cytokines with polymorphisms that have been associated with GC are IL-1β, IL-1Rβ, IL-4, IL-6, IL-10, TNF-α, and TNF-β[93-97]. Based on ethnic backgrounds, two sets of haplotypes for IL-1 β and IL-10 have been related to increased risk for GC[93-95]; specifically, IL-1β-1464G/-511C/-31T and

IL-10-1082G/-819C/952C for Asians and IL-1β-1464C/-511T/-31C and IL-10-1082A/-819T/952T for Caucasians[95].

Canedo *et al*[96] found that IFN-γ receptor 1 -56C/T polymorphism is a relevant host susceptibility factor for GC development associated with *H. pylori* infection. Polymorphism in the promoter region of the gene coding for IL-10 and TGF-β has been also described in the Mexican population in relation to susceptibility to GC[97]. There is a need for extended studies in different populations and in larger patient groups, particularly in regions of Latin America where the burden of GC is more severe. In recent years, NF-κB has been widely studied in inflammation, immunity and cancer, but its roles are still unclear[98-100]. NF-κB is a master transcription factor activated downstream of the TLR and cytokines such as TNF-α and IL-1β[98]. In contrast to the canonical NF-κB pathway, the noncanonical NF-κB activation responds to specific stimuli, including ligands from the TNFR superfamily members such as LTβR, BAFFR, CD40 and RANK[99].

In GC, *H. pylori* is associated with increased expression of the proinflammatory NF-κB[101-104]. It has been shown that the induction of NF-κB mediated by *H. pylori* induces the expression of activation-induced cytidine deaminase, which has been demonstrated to induce nucleotide modifications in the *TP53* gene in gastric cell models[105] and suggests that the accumulation of those *TP53* gene alterations might contribute to the development of gastric neoplasia.

As an example, *in vitro* studies showed that programmed death (PD)-1 is increased among gastric epithelial cells after *H. pylori* infection and its immunosuppressive functions on T-cells may contribute to carcinogenesis[106]. Evidence from small studies observed an up-regulation of PD-1 and programmed cell death-ligand 1 (PD-L1) in human *H. pylori*-related gastric carcinoma[107].

In addition, *H. pylori* has shown other effects within the gastrointestinal epithelium not associated with inflammation but compromising the genome stability, for example, reduced levels of transcripts for DNA mismatch repair (MMR) proteins such as MutS, MutL RAD51, FEN1, POLD1, and LIG1[108-110], and this phenotype might be more severe in cases cagA+ *H. pylori*[110]. However, the deficiency MMR in gastric tumors was recently shown to predict clinical response to pembrolizumab[111], demonstrating also the expansion of antigen-specific T cells reactive to tumor-derived neoantigens, suggesting that further studies are required to understand the interaction between pathogens and genomic features as biomarkers.

CROSSTALK BETWEEN VIRAL INFECTION AND INNATE IMMUNITY ACTIVATION

The EBV is a ubiquitous virus and member of the subfamily of human Gammaherpesvirinae[112] and can infect several cell types, including B-lymphocytes, epithelial cells, and fibroblasts[113]. EBV is the main pathogenic factor for nasopharyngeal carcinoma. However, studies find that EBV infection is also associated with the development of T-cell lymphoma and EBV-associated GC[114]. Although the infection rate of EBV is extraordinarily high, reaching over 90% of the adult population worldwide[115, 116], the incidence rate of EBV-positive GC remains low, representing around 9% of characterized stomach adenocarcinomas[117], these EBV-positive tumors display recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2 and both PD-L1 and PD-L2[5].

The EBV-positive GC has been characterized by an increased expression of PD-L1, and a sustained immune-infiltration, which is indicative of the presence of stable T-cells and supports the use of an immune checkpoint inhibitor for the treatment of this GC subtype[118,119]. In addition, most EBV-positive GCs show MSI which has also been associated with inflammation and local immune activation [120-123]. Previous groups have shown that high density of intra-tumoral or stromal CD8+ T cells with a high percentage of PD-L1 expression seems to be associated with a worse progression-free survival and overall survival[118,120,124]. However, a recent study demonstrated that EBV-positive GC patients treated with immunotherapy showed favorable responses[125], suggesting that viral status represents a potential predictive biomarker for using immune-checkpoint inhibitors; however, the balance between pro-inflammatory and immunosuppressive signals together with a concomitant viral infection requires more studies to clarify its role as a biomarker.

Type I and type II IFN are central to both combating virus infection and modulating the antiviral immune response[126]. The cytokine IFN-γ is mainly produced by T Cell CD4+ and natural killers to activate macrophages. The ligation to its receptor triggers an activation of the Janus-Activated kinases, JAK1 and JAK2, and subsequently the activation of STAT1 and interferon regulatory factor 1 (IRF1). STAT1 and IRF1 are activated by phosphorylation and translocated to the nucleus to regulate the *IFN-γ* gene expression[127].

Crucial to the induction of type I IFN is the recognition of viral PAMPs by PRRs, among which, the cyclic GMP-AMP synthase-stimulator of interferon genes modulates the antiviral response triggered by DNA viruses and retroviruses[128]. In addition, most viruses, including EBV, stimulate innate immune response during primary infection predominantly by activating the expression of TLRs, such as TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9[129]. TLR2 is likely activated by EBV surface glycoprotein gp350 [130] and the nonstructural protein dUTPase[131], while EBV-encoded small RNAs released from EBV-infected cells are detected by TLR3[132]. Furthermore, EBV can activate monocytes and plasmacytoid

DCs through cooperative action of TLR9 and TLR2[133].

Activation of PRRs by EBV-PAMPs triggers JAK-STAT-mediated IFN response and different branches of innate immune signaling including NF-κB pathway; inflammasome activation; and programmed cell death such as apoptosis and necroptosis[134]. However, innate immunity is a double-edged sword as the induction of pro-inflammatory responses and activation of programmed cell death might release a burst of virions and may therefore facilitate the spread of infection[135].

Recently, IFN-γ has been the subject of studies due to its immune role in cancer development, especially in GC. There are several signaling pathways of innate immunity during GC development in which interferon is involved, such as in proliferation, metastasis, and advancement of GC through the upregulation of integrin β3-mediated NF-κB signaling[136]. Serum levels of IFN-γ are elevated in GC which may promote systemic and local responses[137] at the same time, for example: peroxisome proliferator-activated receptor delta, a ligand involved in physiologic processes in cell metabolism, proliferation, and inflammation[138] together with IFN-γ signaling creates an inflammatory tumor-promoting microenvironment enabling villin-expressing gastric progenitor cells transformation and gastric tumorigenesis[139]. Furthermore, natural killer (NK) cells play a role in innate immunity against cancer cells. Lee *et al*[140] reported that IFN-γ produced by the activated NK decreases in GC patients compared with healthy donors. This low level of IFN-γ-NK could be used as a non-invasive biomarker for carcinogenesis in GC[140].

Some reports including Latin American cohorts have shown that concentrations of IFN-γ and IL-10 are significantly higher in GC than in non-oncological cases, and within the GC group, IFN-γ levels are increased at the early stages (I/II) and remain higher in late stage (IV)[141]. Interestingly, increased levels of viral capsid antigen antibodies are significantly associated with elevated serum levels of IFN-γ, particularly in the intestinal type of GC[142]. Therefore, IFN-γ is suggested as a biomarker for assessing GC risk; however, this molecule is known to mediate gastric damage or immune antipathogen responses, as well as the expression of some negative immune checkpoint molecules.

The PD-L1 expression is activated by several cytokines, of which IFN-γ is the strongest[143]. In a melanoma cell line model, PD-L1 has shown to be mainly regulated by the type II interferon receptor signaling pathway through JAK1 and JAK2, several STATs including STAT1/STAT2/STAT3, to converge on the binding of IRF1 to the PD-L1 promoter[144]. Later, Chen *et al*[145] treated with IFN-γ thirty-four cultured human tumor lines, including 18 melanomas (MEL), 12 renal cell carcinomas (RCC), 3 squamous cell carcinomas of the head and neck (SCCHN), and 1 non-small-cell lung carcinoma, and as wildtype control the authors considered isolated peripheral blood monocytes. The results indicated that PD-L1 was constitutively expressed on 1/17 cultured MELs, 8/11 RCCs, 3/3 SCCHNs, and on monocytes; however, the inhibition of STAT1 but not STAT3 was more critical to reduce IFN-γ-induced PD-L1 protein expression on tumor cells[145]. Other authors have provided evidence of a crosstalk between JAK2-STAT1 and PI3K-AKT pathways in response to IFN-γ in lung adenocarcinoma[146]. Transcriptome analysis demonstrated that tumor tissues expressing IFN-γ display gene expression associated with suppressed cell cycle progression and expansion, which was not observed in PD-L1 negative tumors. In lung adenocarcinoma cells, IFN-γ induces the activation of JAK2-STAT1 and PI3K-AKT pathways, showing that the activation of JAK2-STAT1 is responsible for the anti-proliferative effect of IFN-γ, and the inhibition of PI3K downregulates PD-L1 expression and enhances the anti-proliferative effect of IFN-γ[146]. In addition to the cytokine regulation, a lncRNA (long non-coding RNA) named Interferon-stimulated non-coding RNA 1 (INCR1) has been described as a major regulator of IFN-γ signaling in tumors by post-transcriptional modulation of PD-L1 and JAK2 expression[147]. INCR1 is expressed as an antisense RNA from the PD-L1/PD-L2 locus and has been detected in human samples across multiple tumor types, and its levels increase after IFN-γ stimulation, correlating with PD-L1 but not PD-L2 expression[147].

Regarding GC, PD-L1 has shown a wide and very variable range of expression based on technique and cutoff, however, it seems to be absent in non-tumor gastric tissue[148-151]. Imai *et al*[152] showed IFN-γ treatments enhanced the expression of intracellular and membranous PD-L1 expression in GC cell lines. This upregulation of PD-L1 induced by IFN-γ was associated with the JAK-STAT but not the MAPK and PI3K-AKT pathway activation[152,153]. PD-L1 overexpression mediated by IFN-γ is also seen in GC with positive EBV[154]. Polymorphism in PD-L1 related to GC has also been described. PD-L1rs2297136 was positively correlated with a higher proportion of PD-L1 protein and could be employed as a tool of prognosis in GC patients[155,156].

A recent study suggested the role of ISG12a as a tumor suppressor in gastrointestinal tissue[157,158]. ISG12 or interferon alpha-inducible protein 27 promotes β-catenin proteasomal degradation by inhibiting the degradation of ubiquitinated Axin, thereby suppressing the canonical Wnt/β-catenin signaling pathway[158]. Reduced levels of ISG12a were observed in gastrointestinal cancer, such as hepatocellular cancer and GC, and it was associated with an immune-suppressive tumor microenvironment. The authors argue that β-catenin is a transcription factor for PD-L1, and the inhibition of the Wnt/β-catenin signaling by ISG12a makes tumor cells more sensitive to NK cell-mediated killing[157]. Therefore, the balance between the induction or suppression of IFN-stimulated genes[159], such as ISG12a, may accelerate the malignant transformation of cancer cells and lead to a poor prognosis in gastrointestinal cancer[157,160].

NON-BIOLOGICAL FEATURES AS IMMUNE AND PREMALIGNANT MODULATORS

GC is currently accepted as the consequence of a multifactorial process, involving pathogen infection and the virulence of some strains (as *H. pylori*), environmental factors, dietary habits, and host intrinsic factors, as we have discussed above; however, socioeconomic factors, such as education level and occupation, have shown to be determinant during the progression of the premalignant cascade of GC and the patient's outcome after the cancer onset[161-163].

Asia accounts for 71% of GC's worldwide, in which China's incidence is 44.1%. The high incidence in China is marked by the rural population, and their exposure to carcinogens through diet, the environment and the *H. pylori* infection *per se*[164]. The incidence of GC in Europe is heterogeneous; while the highest incidence is in Central and Eastern Europe, the lowest incidence is in Western and Northern Europe, which correlates with a higher detection of *H. pylori* in Eastern Europe compared to Western Europe[165], as well as an observed higher consumption of red and processed meat resulting in an increased risk of GC[166].

After Europe, Latin American countries have shown a high incidence of gastric malignancies. The associated risk factors are infection with *H. pylori*, diet and habits such as smoking, consumption of salt, alcohol and meat, as well as ethnicity and age[167]. Some authors have suggested that Latin America has a close correlation between GC incidence and altitude which is based on the presence of a mountainous geography, such as the Sierra Madre, Cordillera de Centroamerica and the Cordillera de los Andes[168]. However, the mortality and incidence rates for gastric malignancies in the Chilean population is statistically higher than the average rates in the rest of Latin America, becoming the second cause of death from cancer in Chile, affecting 11.6 per 100000 inhabitants and causing around 3000 deaths per year[1]. That incidence seems to correlate mainly with socioeconomic status, Mapuche ethnicity, and age at the primary *H. pylori* infection[169].

Surprisingly, most of the countries with the highest incidence of GC are not those with low incomes (Table 2). In fact, it seems that the vicious circle between precancerous lesions, inflammation and GC onset is caused by the low level of education within the population. A study performed within the Swedish population, that considered the economically active population, showed an increased risk factor of GC in workers engaged in manual-labor occupations and in industry. The statistics were standardized for categories of occupation and adjusted by age, period and region, and confirmed that overall manual-workers and farmers had the highest risk of GC, including male miners and quarry workers[162]. The European Prospective Investigation into Cancer and Nutrition cohort included about 520000 participants mostly aged 35–70 years and, after an average follow-up of 6.5 years, reported 268 cases with adenocarcinoma of the stomach. Higher education was significantly associated with a reduced risk of GC with a hazard ratio (HR) of 0.64 (95%CI: 0.43–0.98) and, as was expected from other reports, that effect was more pronounced for cancer of the cardia (HR: 0.42) as compared to non-cardia GC (HR: 0.66)[163].

A survey to address GC risk factors and endoscopic screening within the North American population showed that ethnicity, cultural habits and immigration patterns are potentially useful to identify high-risk persons from multicultural areas within the United States[170]. The authors identified that dietary habits during teenage years (15-18) and education below high school level may represent signs of risk for GC in older people of foreign birth[170]. Most recently, based on the previous research, a secondary analysis showed that education level was the single most reliable measure of GC risk among three variables of socioeconomic status including, education, income, and occupation, which are the most commonly used for health outcomes such as cancer survival[171]. Similar results were observed in a seroepidemiologic study in Japan where the *H. pylori* positive rate increased at 1% per year for people born after 1950 but was comparatively constant for people with birth dates before 1950[171]. Based on the authors, the apparent decreased prevalence of *H. pylori* post-war was accompanied by the Western-ization of the country and subsequently by a reduction in the frequency of atrophic gastritis and the incidence of gastric carcinoma during the most modern times[171,172].

The infection status in adults is considered to be influenced by socioeconomic status in childhood; however, given the massive improvement in hygiene and the economic environment around the world, it has contributed to the variation in the trends in incidence and death rates of GC among the countries mentioned above. Based on other authors, the education in *H. pylori* eradication and gastric malignancies is largely due to unplanned prevention caused by the widespread adoption of technology and improved manufacturing practices of the food industry. In a similar way, the prevalence of *H. pylori* infection may be reduced owing to improvements in sanitary and housing conditions based on education at early ages by primary schools and in adult life by primary health workers.

Where the intersection between education and immunity is not evident, by intuition a limited knowledge regarding gastrointestinal health and eradication of *H. pylori* infection might dramatically influence the development of GC. Therefore, the inflammatory process induced by a pathogen or even an incipient neoplasm may not receive enough attention, progressing finally to an advanced disease with limited therapeutic opportunities and uncertain outcome for the patient.

Table 2 Gastric cancer incidence and socioeconomic indicators per country

Country	Estimated cases	Crude rate	ASR, world[1]	Cum. risk	HDI classification
Japan	138470	109.5	31.6	9.35	Very high
Korea	28713	56	27.9	6.51	Very high
Brunei Darussalam	55	12.6	13.5	5.93	Very high
Russia	37364	25.6	13.5	3.29	Very high
Chile	4208	22	13.1	4.17	Very high
Lithuania	864	31.7	13	3.4	Very high
Estonia	379	28.6	12.3	3.2	Very high
Latvia	530	28.1	12	3.01	Very high
Portugal	2950	28.9	11	2.99	Very high
Slovakia	1210	22.2	10.7	3.12	Very high
Mongolia	860	26.2	32.5	7.71	High
China	478508	33.1	20.6	5.24	High
Iran	14656	17.4	17.5	6.58	High
Kazakhstan	3357	17.9	15.8	3.47	High
Belarus	2739	29	15.4	3.5	High
Peru	6300	19.1	15.2	5.16	High
Colombia	8214	16.1	12.8	3.61	High
Costa Rica	952	18.7	12.8	4.12	High
Samoa	20	10.1	12.8	3.75	High
Azerbaijan	1453	14.3	12.7	3.42	High
Tajikistan	1301	13.6	23.4	6.96	Medium
Kyrgyzstan	1027	15.7	19.7	5.01	Medium
Cabo Verde	82	14.7	18.4	5.88	Medium
Bhutan	118	15.3	17.7	3.88	Medium
Viet Nam	17906	18.4	15.5	3.57	Medium
Sao Tome and Principe	18	8.2	14.7	2.06	Medium
Myanmar	7235	13.3	13.7	3.58	Medium
Lao	675	9.3	12.9	3.17	Medium
Guatemala	1637	9.1	12.2	3.93	Medium
Turkmenistan	583	9.7	11.8	2.37	Medium
Haiti	1184	10.4	13.5	4.58	Low
Mali	1097	5.4	12.8	2.96	Low
Afghanistan	2149	5.5	12.4	3.18	Low
Zimbabwe	641	4.3	9.4	3.24	Low
Papua New Guinea	474	5.3	9.2	3.17	Low
Rwanda	587	4.5	8.1	1.61	Low
Yemen	966	3.2	7.1	2.68	Low
Senegal	597	3.6	7	1.66	Low
Benin	429	3.5	7	2.29	Low
Mauritania	143	3.1	5.6	1.46	Low

[1]Top 10 countries with the highest age-standardized incidence rates per 100000 inhabitants, per each human development index classification. Source: Globocan 2020, access by https://gco.iarc.fr. ASR: Age-standardized incidence rate; HDI: Human development index.

CONCLUSION

During the transition from premalignant lesions of the gastric epithelium to the final carcinoma, some microorganisms will play a determinant role promoting the neoplastic transformation or contributing with particular tumor phenotype and its heterogeneity, together with different mutagenic agents and genomic aberrations. This review provides an overview on innate immune activation in the context of gastrointestinal malignancies and compiles studies related to TLRs, neutrophils, cytokines and pathogens, as crucial players during the precancerous cascade and the cancer onset, allowing the correlation of those aspects with clinical and socioeconomic variables.

As shown in the graphical abstract, intersections between innate immune response and GC development is driven by common mediators, such as NF-κB and IFN-γ molecular pathways, cellular processes (neutrophil and myeloid cells activation), and activators/inducers (PAMP, damage-associated molecular patterns, tumor-antigens). However, universal risk factors are identified affecting globally any human being which, depending on extrinsic and intrinsic host factors, might facilitate the progression of the precancerous cascade to GC.

Modern therapies such as adoptive cell therapy, vaccines, and especially immunotherapy using checkpoint inhibitors, which have not been mentioned in this work, have shown in clinical trials the role of restoring the balance in favor of the immune system against GC. In the words of Dunn *et al*[173] the observed benefits in GC may be based on (1) Elimination: NK cells and T lymphocytes (helper and cytotoxic) secrete interferon IFN-γ leading to a reduction of angiogenesis and proliferation of cancerous cells; moreover, macrophages and DC secrete cytokines that activate immune cells to phagocytize dead tumor cells; (2) Equilibrium: Residual cancerous cells remain in a dormant state because DC and cytotoxic T cells secrete IFN-γ and inhibitory cytokines (IL-12) suppress them; and (3) Escape: Tumor cells change their features, up-regulating immune checkpoint pathways, which will be transmitted to the daughter cells, therefore escaping immunosuppression and proliferating, along with the apoptosis of the effector lymphocytes[173]. The success of those treatments relies on an efficient immune system, and of course an innate response able to promote priming and mobilization of newly activated immune cells; the presence of pathogens or their PAMPs, as well as molecules derived from the tumor itself will continuously modulate the tumor immune microenvironment. The shape of the tumor will also depend on the host background (genome stability, driver mutations and other gene variants) but in addition, the external environment shaped by the socioeconomic status of each person will determine the final outcome.

Innate immunity is the beginning, and certainly, will be part of the final response against tumors. All these observations require further investigation but it is clear that pathogens like *H. pylori* and EBV need to be assessed in GC based on their role as biomarkers and as potential mechanisms of resistance to therapy.

FOOTNOTES

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REVIEW

Emerging role of non-invasive and liquid biopsy biomarkers in pancreatic cancer

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Abstract

A global increase in the incidence of pancreatic cancer (PanCa) presents a major concern and health burden. The traditional tissue-based diagnostic techniques provided a major way forward for molecular diagnostics; however, they face limitations based on diagnosis-associated difficulties and concerns surrounding tissue availability in the clinical setting. Late disease development with asymptomatic behavior is a drawback in the case of existing diagnostic procedures. The capability of cell free markers in discriminating PanCa from autoimmune pancreatitis and chronic pancreatitis along with other precancerous lesions can be a boon to clinicians. Early-stage diagnosis of PanCa can be achieved only if these biomarkers specifically discriminate the non-carcinogenic disease stage from malignancy with respect to tumor stages. In this review, we comprehensively described the non-invasive disease detection approaches and why these approaches are gaining popularity for their early-stage diagnostic capability and associated clinical feasibility.

Key Words: Non-invasive biomarkers; Cell free biomarkers; Proteomic biomarkers; Liquid biopsy-based diagnostics; Pancreatic cancer biomarkers

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Core Tip: Considering the limited commercial availability of cell free nucleic acids and secreted proteome-based non-invasive biomarkers it is crucial to summarize them for the proper diagnosis of pancreatic cancer and distinguish it from other benign pancreatic diseases. We also highlighted the clinical use of these non-invasive biomarkers in diagnostics. This review will successfully guide readers to address the current issues and aid in the cutting-edge development of biomarkers with higher sensitivity and specificity.

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INTRODUCTION

The global population is severely burdened by the increase in multiple types of cancer. Cancer-related incidence and its associated mortality have emerged as a major threat to global health in developed countries, like the United States, European countries, Canada, and Australia, and developing as well as under-developed countries including India and Southeast Asian countries. Pancreatic cancer (PanCa) is a deadly and emerging type of cancer because of its associated poor survival. Only 20% of patients are eligible for surgery due to the physiologically deep-rooted location and the generalized symptoms caused by the close proximity to crucial vessels. In 2022, the estimated new incidences in the United States alone are 62210 (male) and 32970 (female), and the estimated deaths associated with PanCa are 25970 males and 23860 females.

Per the GLOBACON 2020 data, PanCa is the 12th most common cause of cancer with 495000 new cases across the globe with approximately 47% of the new cases registered in Asia and another major proportion of 28% in Europe. There might be an increase in the incidence by 70% by the end of 2022, which translates into approximately 844000 new cases per year. As documented in the United States population, PanCa-related mortality has relatively increased in men than in women. In the United Kingdom, PanCa is the tenth most frequent cancer type, and it ranks fifth in terms of cancer-related deaths per year. Some risk factors associated with PanCa apart from genetic predisposition include obesity, diet and smoking. PanCa-associated surgery has a high mortality risk making it a more life-threatening disease[1-4]. Based on the demography and ethnicity of patients, the profile of biomarkers and predisposition to particular risk factors can vary[4,5].

LIQUID BIOPSY AND ITS ROLE IN PANCA DIAGNOSIS

Liquid biopsy (also known as a fluid biopsy) is an approach to identify markers released from tumors in the blood or associated body fluids. It includes but is not limited to cell-free DNA (cfDNA), cell-free RNA (cfRNA), cell-free proteins, circulating tumor cells and exosomes[6-8]. Liquid biopsy is rapidly developing and has a highly emerging market with an estimated $5 billion (USD) turnover globally by 2023[9]. DNA is the most dominant approach in terms of biomarker identification from plasma or serum and in terms of availability for clinical practices, which makes cfDNA a lucrative option.

There are multiple factors that make liquid biopsy an attractive option in comparison to tissue biopsy. A major reason is that traditional tissue biopsy fails to identify tissue heterogeneity in depth whereas liquid biopsy does that more efficiently[10]. The reason behind this is based on the concept that various categories of tumor materials are released in the blood irrespective of tumor heterogeneity. Therefore, making the liquid biopsy samples, such as plasma or serum, a cocktail that has tumor markers irrespective of cell type is not present in the case of the traditional biopsy technique. In addition, the exponential growth of next generation sequencing technologies enables the identification of cell free nucleic acids with high sensitivity, which is of great clinical application.

Moreover, traditional tissue biopsy is not applicable in the case of patients that are in an early stage and have not developed a tumor of significant size[11]. Early-stage PanCa is usually asymptomatic, and no operative options are available to examine the tumor, making traditional tissue biopsy non-

functional in this context. For early or advanced diagnosis and disease assessment, liquid biopsy can be a major step forward toward cancer prevention and screening of populations. APAPTEST was the first cancer blood-based test done to identify markers in the case of cervical cancer[12]. Disease-targeted liquid biopsy-based biomarkers can detect patients who have an intermediate chance of developing PanCa such as those having chronic pancreatitis (CP), diabetes or a genetic predisposition to PanCa. It can also distinguish between PanCa and pancreatic diseases that are immune system modulated in their origin.

A crucial requirement is distinguishing autoimmune pancreatitis (AIP) from PanCa. AIP diagnosis, which is an autoimmune disease based on the rise in IgG4 levels in serum, is based on a liquid biopsy-based approach for identification and differentiation from malignancies such as PanCa. Upregulation of IgG4-positive plasma cells in organ tissues followed by increased IgG4 levels in the serum is usually defined as type 1 AIP. On the contrary, type 2 AIP has no connection with IgG4. It demonstrates features in the pancreatic ducts resembling granulocytic epithelial lesions. Type 1 AIP also acts as a pancreatic lesion related to IgG4-related disease[13]. Since both type I AIP and type II AIP are based on identification using non-invasive approaches, the development of these techniques is urgent to support cases where invasive approaches are a less viable option. This approach acts as a powerful alternative to existing diagnostic methods and will be highly beneficial for the identification of cases with aggressive tumor biology and support clinicians to make proper individualized targeted therapy[13-17].

The methylation status alteration in cancer cells is also an important point to consider for disease diagnosis. The nature of methylation based alterations discriminates itself from genetic changes based on tumor tissue type specificity, which is crucial in the case of methylation alterations apart from being more pervasive and prevalent in terms of occurrences[18,19]. Aberrant DNA methylation alterations mostly occur in CpG islands and play a vital role in activating tumor suppressor genes and oncogenes, thereby modulating the genome[20-23]. The approach to identifying cfDNA-based methylation changes is a growing area of interest because invasive approaches are not always feasible and proactive in terms of clinical diagnostics[24,25]. Various reports claimed that in the case of pancreatic ductal adenocarcinoma (PDAC) an altered methylation signature has been observed when tumor tissues were compared with normal tissues, hence directing its implications in biomarker development[26,27].

The neoplastic tendency of the pancreas needs to be identified, and specific non-invasive markers discriminating PanCa from CP or other pancreatic precursor lesions such as pancreatic intraepithelial neoplasia (PanIN), intrapapillary mucinous neoplasia (IPMN) and mucinous cystic neoplasm need to be developed[28]. Luo *et al*[29] documented that cg10673833, a circulating tumor DNA (ctDNA) methylation marker was found to be very sensitive (89.7%) and specific (86.8%) for the detection of colorectal cancer and its associated pre-cancerous lesions. SEPT9 was the first blood-based screening test that the Food and Drug Administration approved for colorectal cancer. Cancer-seek was developed by Tivey *et al*[30]. It is a blood test that detects circulatory protein and cfDNA biomarkers. It is capable of detecting eight cancer types, mostly in their respective advanced stages, with a specificity of 99% and a sensitivity of 69%-98%, which varied based on cancer type[12,20].

PANCA AND ITS ASSOCIATED BENIGN DISEASES

Systemic approaches to develop biomarkers to distinguish PDAC from other precancerous lesions are of great importance as they will also support the early diagnosis of PDAC and substantially improve outcomes. Single gene analysis has its limitation in early detection because it has been observed that the specificity and sensitivity of biomarkers in combination increase more than single biomarkers. Traditional serum-based biomarkers like CA19-9, CA125, and carcinoembryonic antigen (CEA) for PanCa diagnostics are of immense importance, but there is a need for the development of more sensitive and specific non-invasive biomarkers. The sensitivity and specificity of detection are usually much higher when the above three biomarkers are used jointly instead of individually[21,31]. In addition, varied combinations of DNA methylation-based biomarkers are being developed to better understand the complexity of the diseases including both the initiation and neoplastic stages. Concurrently addressing the contribution of immune cells in the cfDNA pool and its associated methylation or hydroxyl-methylation pattern is of great importance as it can be obtained from plasma samples[32-35].

Almost 50 of 100000 people are affected by CP worldwide with an increasing trend, and a CP-associated prolonged inflammatory state enhances tissue conversion to neoplasia. Pancreatic tumor (PT) formation is also increasing along with CP worldwide. Mortality due to PTs is close to 6%. After a diagnosis of CP, it takes almost 10 years before tumor symptoms appear, which is a long latency period with sufficient time for early diagnosis and treatment *via* non-invasive approaches. *PENK* DNA methylation has been observed broadly in precancerous lesions of various grades including extraductal and intraductal PTs and CP along with PanIN, IPMN and mucinous cystic neoplasm. *PENK* methylation alterations increased as the neoplastic tissue grade increased but was absent in the case of AIP and adjacent normal pancreatic tissue[36].

In a study of an 87 gene panel, 6 genes, namely *PRKC, IKZF1, CD1D, KCNK12, CLEC11A* and *NDRG4*, were shown to have clinical ability and potential as a cfDNA diagnostic biomarker for PanCa[37]. In this

study using pancreatic juice taken from PanCa, CP and healthy patients, the authors documented that most of the biomarkers were successful in discriminating patients with PanCa from healthy patients, but only *CD1D* was successful in discriminating patients with CP from PanCa with a sensitivity of 84%[37,38]. A remarkable change in methylation status was documented by Liggett *et al*[39], where they showcased that hypomethylated genes in CP converted to hypermethylated genes in PanCa, suggesting that altered methylation is also a driving reason for neoplasia development in the case of PTs.

A total of 14 genes, namely *CCND2, CDKN2B, DAPK1, MUC2, MYOD1,* promoter A of *MLH1, CDKN1C, ESR1, MGMT PGK1*, the proximal region of the *PGR* promoter, *RARB, RB1* and *SYK*, were successful in distinguishing CP from PanCa based on cfDNA DNA methylation profiling with 91% sensitivity and specificity[38,39]. In a study to identify cell death specific to tissue type by Lehmann-Werman *et al*[40], they observed that *CUX2* and *REG1A* are specific to pancreas tissue type. They also found these genes to be hypomethylated in plasma in both PanCa and CP, but the levels were lowered in other lower neoplastic grade lesions. *CUX2* was more hypomethylated in the case of PanCa and *REG1A* was higher in CP, which gives the ability to discriminate CP from PanCa based on tissue death irrespective of etiology[38,40].

In a study using pancreatic juice samples collected from PanCa and other associated precancerous lesions, six genes were demonstrated to be differentially methylated between CP and PanCa[41]. The six genes, *TFPI2, NPTX2, FOXE1, p16, ppENK* and *CyclinD2*, were able to successfully distinguish between CP and PanCa as CP had lower levels of methylation than PanCa. Similarly, the levels differed amongst healthy tissue and high-risk patients creating a gradient of altered methylation across various stages starting from healthy to precancerous to PanCa samples. It was also able to distinguish between IPMN and PanCa based on cfDNA methylation from pancreatic juice samples. Further, the study also attempted to address the limitations of IPMN diagnostics, which cytology mostly fails[42]. Promoter DNA methylation of the *CDO1* gene was identified to be involved in early-stage PanCa based on cytological experiments. cfDNA methylation analysis of *CDO1* from PanCa, CP and AIP patients showed that significant promoter methylation was associated with PanCa but was absent in CP and AIP [43]. In another study, *CD1D* showed cfDNA methylation when analyzed in pancreatic juice samples of PanCa [area under the curve (AUC): 0.92], but no methylation was documented in benign diseases[21,43]. In a study by Humeau *et al*[44], they reported that urine and sputum are also suitable alternatives to plasma for cell-free nucleic acid biomarker development. The utility of other material such as urine as a source of non-invasive testing needs further data and investigation.

MicroRNA (miRNA), namely hsa-miR-23a and hsa-miR-23b, have been documented to be overexpressed in IPMN patient saliva, which makes it a suitable precancerous lesion biomarker. It was also observed that hsa-miR-210 along with let-7c signals may be leveraged from saliva in distinguishing CP from healthy subjects[32,44]. Su *et al*[45] concurrently conducted a study using serum as a sample source and observed that miR-877, miR-3201, miR-890, miR-602 and miR-16-2-3p were successful in distinguishing low-risk and high-risk PDAC[32,45]. Irrespective of this development, there is still a hindrance in using miRNAs in clinical diagnostics due to reproducibility issues and variable expression of miRNAs. However, substantial work is ongoing in this area[32].

In a study to differentiate PDAC from CP by Bosch *et al*[46], it was documented that the the presence of a high level of soluble AXL (sAXL) was associated with lower overall survival in the case of PDAC. When identified from plasma, a higher level of sAXL was documented in PDAC than in CP and healthy controls, and they concluded that it is a potential biomarker for discriminating CP from PDAC and useful in identifying the onset of PDAC by its increasing level during monitoring. The findings were also validated in murine models. sAXL was also found to be overexpressed in IPMN, which is a pre-PDAC stage[46].

Weeks *et al*[47] conducted research where they studied the urine proteome differences amongst PDAC, CP and healthy individuals. They noticed 101 proteins that were significantly altered in PanCa and CP. S100A9 was found to be downregulated in PanCa but overexpressed in CP. Annexin A2, CD59 and gelsolin proteins were also found to be dysregulated in PDAC samples[47]. An isotope-coded affinity tag tended to successfully distinguish between PanCa and CP based on quantitative protein expression[41]. In this review, we documented the important cell free biomarkers that are vital in the early-stage detection of PanCa and are able to distinguish PanCa from its non-malignant precursors.

CFDNA AND ITS ASSOCIATED SECRETED PROTEOME

cfDNA in PanCa and its association with benign pancreatic diseases

First reported in 1948 by Mandel and Meta[48], cfDNA is undoubtedly the most dominant form among the liquid biopsy-based approaches and when released by tumor is also a ctDNA. Leon *et al*[49] in 1977 introduced cfDNA levels, which were found to be notably more advanced in malignancies than in healthy individuals thereby showcasing its role in carcinogenesis. Similar observations were made in 1983 with PDAC patients, and it was noted that cfDNA levels were higher in PDAC patients than in CP and acute pancreatitis[50]. The origin of cfDNA in the blood can be due to release from tumors, apoptosis or necrosis and other active cellular secretions. A major factor leading to the rise in cfDNA

studies in cancer management is the half-life of cfDNA, which is around 16 min-2.5 h, and that it can be monitored for overall disease progression and associated clinical prognosis with medical treatment (Table 1)[12,49,51].

Cancer diagnosis and treatment face a major setback from recurrence of the malignancy that cannot be ideally monitored using traditional techniques due to the lack of proper tumor formation and its associated diagnostic techniques. Postoperative or post-chemotherapeutic analysis of ctDNA in the blood can also be referred to as minimal residual disease (MRD) analysis. Minimal residual disease analysis cannot be ideally performed using imaging and traditional biopsy techniques, and the analysis using cfDNA/ctDNA is a great advance to measure tumor recurrence (Table 1)[12,52].

In terms of aberrant DNA methylation, which is also a common observation in PDAC patients, it has been documented that a change in methylation levels has been observed in various stages of dysplasia [53,54]. This indicates that the level of cfDNA methylation across various pre-PanCa lesions to PDAC is altered providing a candidate opportunity to track PDAC progression. In 2009, Melnikov *et al*[55] established the use of cfDNA methylation in PDAC diagnosis from 30 plasma samples. This was completed using microarray technology where they identified that the promoter region was hypomethylated in five genes with a sensitivity and specificity of 76% and 59%, respectively[55]. A crucial step in PanCa treatment is staging as that provides a proper clinical treatment approach and is associated with DNA promoter hypermethylation. Henriksen *et al*[56] reported that the use of cfDNA promoter hypermethylation as a prognostic biomarker in PDAC staging, which is based on the hypothesis that cfDNA methylation is stage-specific, can help in stage classification. Henriksen *et al*[56] analyzed a set of 28 genes in PDAC plasma samples. They developed the first prognostic prediction model using cfDNA hypermethylation targeting PDAC staging. They separated cluster cases with and without distant metastasis. The researchers were also able to document significantly higher numbers of hypermethylated genes clustering in stage IV PDAC than in earlier stages amongst which *BNC1* was the most significant gene of interest (Table 1)[25,27,56].

A crucial challenge in cell free nucleosome identification for the differentiation of benign pancreatic disease from PanCa is a nonspecific rise of cell free nucleosome levels in the serum of patients while quantifying the cell free nucleosome. Bauden *et al*[36] identified the cell free nucleosome epigenetic alterations specific to disease type and stages by isolating intact nucleosomes and then quantifying them using an enzyme-linked immunosorbent assay. They were successful in quantifying five serum cell free nucleosome diagnostic biomarkers with an AUC of 0.95, which differentiated PanCa from a healthy pancreas. The epigenetic profiles of cell free nucleosomes often differ between individual patient serum samples based on cancer and healthy populations. Understanding that the epigenetic changes usually occur at the onset of the neoplastic transformation stages, those already in pre-neoplastic stages may represent cell free nucleosome profiles that can act as possible cell free biomarkers for the early detection of cancer (Table 1)[36].

Role of cfDNA as a diagnostic and therapeutic biomarker

Tumors in the pancreas can occur in tissues that are hard to isolate due to their depth of location and also small tumor size can be a limitation. Therefore, in this context blood-based cell free biomarkers are of high clinical importance[56]. Melnikov *et al*[55] investigated that they developed the MethDet56 test which contained screening of 56 gene promoters from PDAC plasma samples that identified five promoters namely *CCND2, SOCS1, THBS1, PLAU* and *VHL* with significant aberrant DNA methylation and with sensitivity and specificity of 76% and 59% respectively. These are initial biomarker development work showcasing that blood-based biomarkers could be highly valuable in diagnosis and prognosis if further studied and developed[55]. In a study by Sato *et al*[57], three out of six genes, namely *CLDN5, SFRP1* and *NPTX2*, previously validated in tissue samples were found to be differentially methylated in pancreatic juice samples collected from PTs[38,57]. However, Matsubayashi *et al*[42] concluded that five genes that were previously validated by solid biopsy failed were not replicated in liquid biopsy. Although the genes *TFPI2*, *PENK, CCND2, NPTX2* and *FOXE1* were successfully able to discriminate PanCa from healthy tissue, the analysis failed to discriminate CP from PanCa or healthy tissue based on cfDNA methylation (Table 1)[42,55].

Yi *et al*[58] interestingly found the use of *BNC1* and *ADAMTS1* as novel serum-based biomarkers for early PanCa detection. The same research group concluded that low frequencies of these two genes were observed in pancreatitis, and there was a significant methylation difference between PanINs and PanCa. *BNC1* could still be detected in PanIN-1, but *ADAMTS1* could only be detected significantly in invasive PanCas[58]. Li *et al*[59] reported in an analogous study that *BNC1* and *SEPT9* were found to be highly methylated in PanCa and were suggested to have a significant role in PanCa diagnostic cfDNA biomarker development from plasma. A limitation of the study was that cfDNA methylation level in plasma was also found to be elevated in other benign diseases such as CP, acute pancreatitis and other lesions as these are risk factors leading to PT formation. This suggests a possible role of methylation changes in driving the progression of benign lesions to metastatic PanCa[60].

Global epigenetic alterations in 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) work as standalone entities in carcinogenesis[61]. Cao *et al*[62] concluded in this context that 5mC as well as 5hmC work as effective cfDNA in PDAC diagnostics and that when 5mC-5hmC work as an integrated system it provides an enhanced diagnostic power. As reported by Guler *et al*[33], PanCa early detection

Table 1 Cell free DNA involved in pancreatic cancer diagnostic and prognostics

Target candidate	Up/down	Sample	Potential value
CEA mRNA	Up	Whole blood	D
EGFR mRNA	Up	Serum	D
4GnT mRNA	Up	Whole blood	D
COL6A3 mRNA	Up	Serum	D/P
Mir-155	Up	Plasma	D
miR-196a	Up	Plasma	D
miR-21	Up	Plasma	D
miR-210	Up	Plasma	D
miR-155	Up	Pancreatic juice	D
miR-21	Up	Plasma	D
miR-196a	Up	Serum	D/P
miR-200a	Up	Serum	D
miR-200b	Up	Plasma	D
miR-210	Up	Plasma	D
miR-18a	Up	Plasma	D/T
miR-16	Up	Plasma	D
miR-196a	Up	Plasma	D
miR-185	Up	Serum	D
miR-191	Up	Serum	D
miR-20a	Up	Serum	D
miR-21	Up	Serum	D/P
miR-24	Up	Serum	D
miR-25	Up	Serum	D
miR-99a	Up	Serum	D
miR-1290	Up	Serum	D
miR-221	Up	Plasma	D/T/P
miR-375	Down	Plasma	D/T/P
miR-375	Up	Plasma	D
miR-27a3p	Up	Whole blood	D
miR-196a	Up	Serum	D
miR-196b	Up	Serum	D
miR-205	Up	Pancreatic juice	D/P
miR-210	Up	Saliva	D/P
miR-492	Up	Plasma	D/P
miR-1427	Up	Plasma	D/P
miR-22	Up	Plasma	D
miR-642b	Up	Plasma	D
miR-885-5p	Up	Plasma	D
Multigene index	Up	Whole blood	D
miR-483-3p	Up	Plasma	D
MIR-21	Up	Serum	D

MIR-210	Down	Serum	D
MIR-155	Up	Serum	D
miR-222	Down	Serum	D
miR-203	Down	Serum	D
miR-132	Down	Serum	D
miR-212	Down	Serum	D
miR-96	Down	Serum	D
mi-126	Down	Serum	D
mi-217	Down	Serum	D
Let-7	Down	Serum	D
miR-144	Down	Serum	D
miR-148	Down	Serum	D
miR-34a	Down	Serum	D
miR-3548	Up	Serum	D

CEA: Carcinoembryonic antigen; D: Diagnostic; P: Prognostic; T: Therapeutic; miR: MicroRNA.

is suitable using 5hmC-based detection techniques, and a regression model showed an AUC of 0.919 encompassing a highly variable gene set. The low cfDNA methylation observation in the case of PDAC was consistent with other studies, and it corresponded to *KRAS* mutation increase in malignancy. PDAC 5-hmC increase was observed at the transcription start site and 3' untranslated region, but a decrease was seen in the promoters[33]. cfDNA-based *KRAS* mutation identification is usually a good indicator of neoplasia though with limited specificity. *KRAS* remains a widely used gene for the early stage detection of PanCa. Other gene mutations, such as *DNMT3A, TP53, GNAS, JAK2* and *BCORL1*, remain alternative ctDNA-based biomarkers[24,63-65]. However, it is clear that analysis of somatic genes in ctDNA provides limited sensitivity in detecting early-stage tumors (Table 1).

Cell free protein (secreted proteome) in PanCa and its associated benign pancreatic diseases

The secreted proteome often acts as a suitable diagnostic biomarker, but currently only CA19-9 is a Food and Drug Administration-approved biomarker for PDAC diagnosis. It has substantial drawbacks and lacks a proper proteomic dataset for PanCa. Often false negative CA19-9 results are given by patients with *FUT3* mutations, and false positives are also observed in non-malignant CP patients as CA19-9 is usually very highly overexpressed[46].

The plasma-free amino acids (PFAAs) index is growing in popularity as a suitable approach for the diagnosis of PanCa. A study to compare the PFAAs index between PanCa and healthy controls by Fukutake *et al*[66] found PFAAs as a significant biomarker capable of distinguishing PDAC. The receiver operating curve AUC developed index was demonstrated to suitably discriminate PanCa from controls. PFAAs are modulated by metabolic changes, in particular in organ systems based on disease induction. As a result, most metabolomic studies consider the analysis of the PFAAs index[66]. Sources of protein biomarker analysis are usually blood and pancreatic juice, but it can also be validated from tissue samples. Wu *et al*[21] documented that altered levels of proteins, namely GPC1, PFAA, OPNT + TIMP-1, CPA4, MUC5AC and C4BPA, were observed in the serum of PanCa patients. They also documented that GPC1 level was significantly higher in PanCa patients than in patients with benign pancreatic diseases such as CP or AIP. It was also documented that serum levels of cell free CPA4 were significantly higher in PanCa than in healthy controls with a parallel observation that C4BPA was comparatively more sensitive than CA19-9. A crucial observation was based on PFAA where it was revealed that PFAA levels correlated with PanCa staging and that can be used for pathological PanCa staging. A significantly high level of serine in plasma was also documented along with a significant decrease in threonine levels in PanCa patient plasma (Table 2)[21,66,67].

Proteomic techniques are gaining a lot of attention for studying protein expression in PanCa. Urine as a source for the non-invasive analysis of biomarkers has gained attention. Advantages of using urine are: (1) Common proteins such as albumin are less abundant; (2) Sample processing is simpler (the plasma secreted proteome (secretome) is much more complex than the urine proteome); (3) Urine is more thermostable; and (4) 49% of proteins are soluble products in urine obtained from glomerular filtration of lymphatic fluids (indicates that various external sources contribute towards the pool of the urine proteome and its protein components). Similar studies have been completed in breast and ovarian cancers; and it is likely to remain an active area of investigation (Table 2)[47,68,69].

Table 2 Cell free protein (secreted proteome) involved in pancreatic cancer diagnostic and prognostics

Target candidate	Up/down	Sample	Target	Pathway	Potential value
ADAMTS1	Up	Plasma	ADAMTS1	Methylation	D
BNC1	Up	Plasma	BNC1	Methylation	D
CDKN1C	Up	Plasma	CDKN1C	Methylation	D
MLH1	Down	Plasma	MLH1	Mutation	D
PGR (prox)	Up	Plasma	PGR	Cell signaling	D
SYK	Up	Plasma	SYK protein, mRNA	Cell signaling	D
CCND2	Up	Plasma	Cyclin D2	Cell signaling	D
ALX4	Up	Plasma	Transcription factor	Mutation	P
APC	Down	Plasma	APC	Mutation	P
BMP3	Up	Plasma	TGFβ	Cell signaling	P
BNC1	Up	Plasma	BNC1	Cell signaling	P
BRCA1	Up	Plasma	BRCA1	Mutation	P
CDKN2B	Up	Plasma	Ink4, p16, p14	Cell signaling	P
CHFR	Up	Plasma	E3 Ubiquitin-protein	Cell signaling, check point	P
ESR1	Up	Plasma	ESR1, Transcription factor	Cell signaling	P
EYA2	Up	Plasma	EYA2	Cell cycle	P
GSTP1	Up	Plasma	GSTP1	Cell signaling	P
HIC1	Up	Plasma	HIC ZBTB TRANSCRIPTIONAL RECEPTOR 1	Cell signaling	P
MEST1v2	Up	Plasma	Alpha/Beta hydrolase superfamily	Cell signaling	P
Foxe1	Down	Pancreatic juice	Foxe1	Methylation	D
MGMT	Up	Plasma	MGMT	Methylation	P
MLH1	Up	Plasma	MLH1	Mutation	P
NPTX2	Up	Pancreatic juice	NP, synaptic proteins	Cell signaling	P
NEUROG1	Up	Plasma	NEUROG1	Cell signaling, regulatory network	P
RARB	Up	Plasma	RARB	Cell signaling	P
RASSF1A	Down	Plasma	RAS effector protein	Methylation	P
SFRP2	Up	Plasma	SFRP2	Wnt signaling	P
SEPT9v2	Up	Plasma	Septin9	Cell cycle	P
CDID	Up	Pancreatic juice	CDID	Methylation	D
PRKC	Up	Pancreatic juice	PRKC	Methylation	D
IKZF1	Up	Pancreatic juice	IKZF1	Methylation	D
KCNK12	Up	Pancreatic juice	KCNK12	Methylation	D
CLEC11A	Up	Pancreatic juice	CLEC11A	Methylation	D/P
NDRG4	Up	Pancreatic juice	*NDRG4* promoter	Methylation	D
SST	Up	Plasma	SST	Downstream signaling, transcriptional regulation	P

TFPI2	Up	Pancreatic juice	TFPI2	Gene silencing, cell adhesion plasmin signaling	P
TAC1	Up	Plasma	TAC1	Gpcr downstream, signal transduction	P
VIM	Up	Plasma	Vimentin	Cell signaling	P
WNT5A	Up	Plasma	WNT5A	Wnt signaling	P
PENK	Up	Pancreatic juice	Proenkephalin	Apoptotic, downstream signaling	
DCC	Down	Plasma	Netrin-1 Receptor	Mutation	D
P16	Down	Pancreatic juice	CDKN2A	Mutation, cell cycle regulation	D
P14	Down	Plasma	CDKN2A	Cell cycle	D
DNMT1	Up	Plasma	DNMT1	Mutation	D
DNMT3A	Up	Plasma	DNMT3A	Methylation	D
DNMT3B	Up	Plasma	DNA	Methylation	D

D: Diagnostic; P: Prognostic.

Role of secretome as a diagnostic and therapeutic biomarker

Cell free nucleic acid alone or combined with the secretome is likely to enhance sensitivity in PanCa diagnosis, and it remains an area of high interest in the field. Research often claims that patients with some signature of circulating proteins ideally overlap with circulating DNA. The main issue is that cfDNA and cell free proteins are most often seen in advanced stages than in early stages[63]. A secretome study to identify cell free proteins as biomarkers in PanCa is usually dominated by the urine proteome. In a study by Poruk *et al*[70], the researchers validated a pair of biomarkers to distinguish between PDAC and healthy controls as well as distinguishing it from CP. This group also focused on the ability of the biomarker to identify the early onset of the disease. The pair of biomarkers were OPN and TIMP-1, and they were found to be uniformly overexpressed in the case of PDAC. Due to their tendency to be available as the secretome in blood, they were easy to detect and were shown to be a useful tool for diagnosis. OPN particularly acts as a more advanced prognostic biomarker by itself[70-72]. Urine samples are also a reliable source of cell free biomarker isolation and identification.

A three-protein biomarker panel was developed from urine samples of PDAC patients and were assayed using GeLC/mass spectroscopy (MS)/MS and validated using an enzyme-linked immunosorbent assay. The candidate protein biomarkers were namely LYVE-1, TFF1 and REG1A, and they provided an AUC of 0.89. These biomarkers were able to distinguish between early-stage PDAC and advanced-stage PDAC and were almost consistent with CA-19 levels that were documented in regard to every PDAC stage. The panel was also able to distinguish between PDAC and CP based on the higher levels of biomarkers in PDAC than in CP as well as PDAC stage I-II. The concentrations of these proteins were significantly higher than CP, which generated a clear demarcation between these groups [73].

Kaur *et al*[74] reported MUC5AC as a suitable biomarker candidate for PanCa diagnosis based on its capability to differentiate between PanCa, CP and AIP. Another urine-based diagnosis study claimed that neutrophil gelatinase-associated lipocalin can provide valuable information on the onset of PanCa identification[21,74]. Considering that pancreatic juice collection is an invasive process, the parallel study was completed studying the role of ARG2 as PanCa biomarker, which was specific in targeting the bile component for PanCa early-stage detection. sLR11, which is present in the bile of PanCa cases, was able to discriminate between PanCa and healthy controls[75]. A panel of CA19-9, CA 242, CA 125 and CEA obtained from serum showed a sensitivity and specificity of 90% and 94%, respectively[76,77]. A novel nucleosome marker panel developed by Bauden *et al*[36] showed better sensitivity than CA19-9 in discriminating PanCa from a healthy control. The markers were *H3K4Me2, H2AZ, H2AK119Ub, H2A1.1* and 5mC[36]. Additionally, as documented by Melo *et al*[78], the absolute prediction (AUC = 1.0) of PanCa using glypican 1 on exosomes identified by MS showed utility as a biomarker (Table 2).

cfRNA in PanCa and its association with benign pancreatic diseases

Amidst the traditional serum-based biomarkers like CA19-9, CA125 and CEA for PanCa diagnostics, there is an immense need for the development of more sensitive and specific non-invasive biomarkers. Sensitivity and specificity are usually higher when the three are used jointly (Table 3)[21,31].

Uncontrolled proliferating cancer has a high mortality rate, and treatment and diagnosis are also difficult due to the lack of screening imaging modalities and specific biomarkers. Emerging evidence of biomarkers generated from tumor cells contains a high quantity of RNA in the bloodstream that block

Table 3 Cell-free RNA involved in pancreatic cancer diagnostic and prognostics

Target candidate	Up/down	Sample	Target	Pathway	Potential value
ERBB2	Up	Plasma	Ch 12, *KRAS*	Mutation	D
CDKN2A	Down	Plasma	Gene, Ch 9p21	Mutation	D
SMAD4	Down	Plasma	TGFβ	Cell signaling	D
TP53	Down	Plasma	P53 protein	Mutation/cell signaling	D
EGFR	Up	Plasma	Exon 19, 21	Cell signaling/mutation	D
PBRM1	Down	Plasma	SWI/SNF ch remodeling comp	Mutation, immune checkpoint blockade	D
KMT2D	Down	Plasma	FOX1-miR-1224, HIF/GATA5-miR-133a	Mutation	D
RNF43	Down	Plasma	RNF43PROTEIN	Wnt signaling	D
TP63	Down	Plasma	P63 protein	Cell signaling	D
MTOR	Up	Plasma	mTOR	mTOR signaling	D
NRAS	Up	Plasma	*NRAS*	Mutation	D
HRAS	Up	Plasma	*HRAS*	Mutation	D
BRCA1	Down	Plasma	BRCA1	Signaling	D
BRCA2	Down	Plasma	BRCA2	Signaling	D
PALB2	Up	Plasma	PALB2	Signaling/mutation	D
GPC1	Up	Serum	MAPK, PI3K-AKT-mTOR, Hedgehog signaling	Cell signaling	D
PFAA	Up	Serum	MAPK, PI3K-AKT-mTOR, Hedgehog signaling	Cell signaling	D
OPNT+TIMP-1	Up	Serum	MAPK, PI3K-AKT-mTOR, signaling	Cell signaling	D
CPA4	Up	Serum	MAPK, mTOR, Hedgehog signaling	Cell signaling	D
MUC5 AC	Up	Urine/pancreatic juice	MAPK, PI3K-AKT-mTOR, Hedgehog signaling	Cell signaling	D/P
C4BPA	Up	Serum	MAPK, PI3K-AKT-mTOR, Hedgehog signaling	Cell signaling	D
OPN	Up	Serum	OPN	Cell signaling	P
TIMP-1	Up	Serum	TIMP-1	Cell signaling	D
NGAL	Up	Urine	Receptor binding protein	Cell signaling	D
ARG2	Down	Pancreatic juice	Beta cell function	Cell signaling	D
LYVE-1	Up	Urine	Receptor binding glycoprotein		D
TFF1	Up	Urine	Regulation of transcription factor	Promoter modeling	D
REG1A	Up	Urine	REG1A protein	Cell signaling	D

NGAL: Neutrophil gelatinase-associated lipocalin; miR: MicroRNA; D: Diagnostic; P: Prognostic.

RNases in blood. They also showcase sufficient levels for quantitative analyses[79]. Living cells consistently release RNA, which is encapsulated inside big lipoprotein complexes, namely exosomes/microvesicles (MVs). RNA that is obtained from dead or dying cells found from blood is connected with apoptotic bodies and protein complexes[80].

Exosomes range from 40-140 nm in diameter and are made of lipoprotein membranous vesicles of endocytic origin. They are formed from the fusion of multivesicular bodies with the plasma membrane. They are released into extracellular spaces thereafter[81]. MVs are much larger than exosomes and have a diameter ranging from 100-1000 nm. They have heterogeneous morphologies, and they originate from the plasma membrane by budding with the extracellular space.

Apoptotic bodies are morphologically MVs that are heterogeneous in shape, spanning 50-500 nm in diameter, and contain organelles. They are released *via* outward protrusion from the plasma membrane

during the late phase of apoptosis[82]. Several studies have demonstrated that tumors specifically secrete exosomes or MVs, which contain specific miRNAs. However, Arroyo *et al*[83] stated that miRNAs in circulation are principally found in the Ago2 ribonucleoprotein complex and not vesicles. This indicates that detected miRNAs are derived mostly from apoptotic and necrotic processes, which occur frequently in tumor cells. These circulating RNAs are found in the plasma or serum of patients and are upregulated when compared to healthy patients.

A significant amount of RNA biomarkers have been shown to be circulating miRNAs and are mostly tissue specific. Some reports found tumor-specific upregulation of various types of small noncoding RNAs (ncRNAs), such as small nuclear RNAs, Piwi-interacting RNAs and long ncRNAs (lncRNAs) like HOTAIR and MALAT1. They act as diagnostic and prognostic markers in the serum or plasma of cancer patients[84,85]. Amidst that, some have reported alterations in gene expression according to disease progression, which discriminates between a chronic inflammatory state and carcinoma (Table 3)[86,87].

Funaki *et al*[88] detected *CEA* mRNA by real-time reverse transcription PCR in the whole blood of PanCa patients. Clarke *et al*[89] also detected *EGFR* mRNA in the serum and detected alpha 1,4-N-acetylglucosaminyltransferase mRNA in the mononuclear cell fraction of peripheral blood from PanCa patients. Kang *et al*[90] further demonstrated that serum-type collagen mRNA acts as good marker of PanCa. It undergoes tumor-specific alternative splicing, which is expected to result in high specificity (Table 3).

In PanCa, the tumor suppressor genes *p53*, *p16* and *SMAD4* are rendered inactive by the miRNAs, which are produced only in tumor tissues[91]. In pancreatic cell lines and tissue, miR-21 is increased, which drastically lowers the survival rate. In the *KRAS* (G12D) animal model, it has been reported that miR-21 overexpression is the lesion onset point that leads to tumor growth. miR-155 is also overexpressed in PanCa. This aids in the development of tumors. Decreasing the expression of miR-155, *MT1-MMP, KRAS,* and *EGFR* inhibits cell growth. miR-221 is also upregulated in PanCa and promotes distant metastasis as well as unresectable tumors[92]. In approximately 50%-70% of cases of PanCa in humans, the *p53* gene has a point mutation. Hypoxia with malnutrition trigger stress signaling in cells that upregulates *p53*, which increases the expression of numerous genes, including *MIR-107*, *MIR-34a/b/c* and *MIR-34* (Table 3)[15,65,86].

Role of cfRNA as a diagnostic and therapeutic biomarker

According to some reports, miRNA dysregulation has been reported in feces, urine and saliva, which are easy to obtain by non-invasive methods. The levels of miR-143, miR-223 and miR-30 were seen to be higher in the urine of patients with stage I disease. The combination of miR-143 with miR-30 showed very high sensitivity and specificity of 83.3% and 96.2%, respectively. Yang *et al*[93] reported that the levels of miR-21 and miR-155 were much higher in PanCa patient stool when compared with healthy controls. Recently studies revealed that salivary miRNAs were stable because of the protection of exosomes or protein complexes, thus highlighting promising roles as diagnostic markers. *SNHG15* serum expression exerts an average diagnostic value (Table 3)[94].

Liao *et al*[95] found that plasma snoRNA *SNORD33/66/76* might serve as a diagnostic biomarker for non-small cell lung cancer (NSCLC) using comprehensive next generation sequencing analysis. Wang *et al*[96] detected the expression of miRNAs in the plasma of patients with PDAC and identified miRs-21, miR-210, miR-155 and miR-196a. They are upregulated in PanCa tissue and cell lines as candidate biomarkers, and miR-200a/b, miR-18a, miR-221 and miR-196a/b have been found to be upregulated in the serum/plasma in parallel with cancer tissues by comprehensive sequence and microarray analysis (Table 3)[96,97].

Kishikawa *et al*[86] suggested that some miRNAs, such as miR-16, miR-223 and let-7, are highly and constantly expressed in the serum/plasma and are correlated with the number of blood cells. Tang *et al*[98] found higher levels of three lncRNAs, long intergenic non protein-coding RNA 1627 (LINC01627), LINC01628 and ERICH1 antisense RNA 1 (ERICH1-AS1; also known as DLGAP2), in the plasma from patients with NSCLC than in samples from healthy individuals after microarray assay. They proposed a potential diagnostic signature for NSCLC based on these lncRNAs. Anfossi *et al*[99,100] suggested that urine levels of miR-148a and miR-375 have been found to be significantly downregulated and upregulated, respectively, in patients with prostate cancer and enabled patients with prostate cancer to be distinguished from healthy individuals (Table 3).

Higher levels of expression of hsa-miR-106a, hsa-miR-125a-5p, hsa-miR-129-3p, hsa-miR-375, hsa-miR-205, hsa-miR-29b, hsa-miR-21 and hsa-miR-7 in histopathology as well as in cytology samples using real-time reverse transcription PCR act as valuable diagnostic biomarkers in different types of cancer[101]. Also, miR-21, miR-210, miR-155 and miR-196a, which have been upregulated in PanCa tissue and cell lines, have been identified by Wang *et al*[96] as candidate biomarkers. A possible diagnostic biomarker for malignant melanoma is miR-221. Li *et al*[103] found that patients with high serum miR-221 levels had significantly worse 5-year relapse-free survival (12.5% *vs* 45.2%; $P = 0.008$). Increased circulating levels of miR-221 allowed patients with melanoma to be discriminated from healthy individuals ($P = 0.0001$) (Table 3)[103].

Rac1 downregulation leads to the switching of the MKK4-JNK-c-Jun pathway, which silences miR-124. In response to TGF, miR-200a and miR-205 are downregulated in PDAC during epithelial-mesenchymal transformation (EMT), while miR-21 overexpression is identified as the lesion initiator

that drives tumor growth in a *KRAS* (G12D) animal model. miR-155, which is elevated in PanCa, aids in the development of tumors.

Karandish *et al*[104] suggested that stress signaling in cells induced by hypoxia and starvation upregulate *p53* and activate the expression of several genes, such as *MIR-107*, *MIR-34a/b/c* and *MIR-34*. Mutation of *p53* mediates transcription of *MIR-130b* and *MIR-155*, which modifies the expression of the corresponding target genes (*ZEB1* and *ZNF652*). This leads to cell proliferation and invasion in several cancers. *ZEB1* has been shown by Wellner *et al*[106] to control the activation of EMT in pancreatic cancer stem cells and to suppress the production of the miR-200 family[104]. In addition, *p53* mutation impairs the maturation of miR-145 and miR-16-1 causing cell proliferation, invasion and migration in PDAC (Table 3).

As a long intergenic non-protein coding RNA, *p53* induced transcript (Linc-pint) is ubiquitously expressed in humans and acts as a direct *p53* transcriptional target. Plasma lncRNA Linc-pint was significantly decreased in patients with PanCa, carcinoma of the ampulla of Vater and cholangiocarcinoma compared with healthy volunteers[108]. Therefore, Linc-pint might be used for identifying the cause of malignant obstructive jaundice and helping to trace the origin of the cancer. Some of these results indicate that low plasma Linc-pint expression could serve as a minimally invasive biomarker for early PanCa detection and that low Linc-pint levels in PanCa tumors could be used for predicting patient prognosis(Table 3)[103].

Prior studies revealed that upregulation of chromatin-interacting lncRNA along with MEG3 inhibited PanCa cell proliferation with the activation of *p53*. Additionally, lncRNA-NUTF2P3-001 contributed to PanCa proliferation and invasion by activating the miR-3923/KRAS signaling pathway. Moreover, LOC389641 promoted the progression of PDAC and increased cell invasion by regulating E-cadherin with the possible involvement of *TNFRSF10A* (Table 3)[109].

The d-signature comprises eight extracellular vesicle long RNAs, including *FGA, KRT19, HIST1H2BK, ITIH2, MARCH2, CLDN1, MAL2* and *TIMP1*, for PDAC detection, and the d-signature could distinguish between CA19-9 negative PDAC cases and healthy controls, thus complementing the use of CA19-9 in PDAC detection. It could also distinguish PDAC from CP with high accuracy. Circ-LDLRAD3 was found to be increased in PanCa tissues as well as in plasma samples. It was markedly related to lymphatic invasion, along with venous invasion and metastasis. Circ-LDLRAD3 has not been reported as an ideal standalone biomarker but may be in combination with CA19-9[110].

The circRNAs *PDE8A* and *IARS* are present in the plasma exosome. They were upregulated along with a concurrent association with prognosis and progression of PanCa. They were also likely to be promising biomarkers in the detection of early-stage PanCa. When compared with the healthy controls, circ-001569 levels were higher. Aberrant levels of *CPA4, PFAA, MUC5AC, GPANCA1, OPNT* + *TIMP-1* and *C4BPA* were frequently reported in serum samples of PanCa patients. In pancreatic juice samples, the higher regulation of ARG2 signifies its role as a biomarker for PanCa diagnosis. Neutrophil gelatinase-associated lipocalin in urine also provides some clue for early diagnosis of PanCa (Table 3)[111,112].

Role of immunological treatments in PanCa and non-malignant diseases

The lack of immune and targeted molecular therapies is a reason for treatment failure and lower survival rates[113]. The reason behind less efficient immune therapies in PanCa is unclear. Usually, PTs are surrounded by thick desmoplastic stroma making targeted treatments less effective. Other reasons of immune therapy failure are chemokine-initiated barring of T cells followed by comparatively faulty antigenicity when compared to other solid tumors[114]. A model states that the systemic progression of PDAC development initiates from PanIN1 to PanIN3 and finally leads to invasive PDAC. This occurs when high progressive deposition of desmoplastic stroma and alteration of leucocyte infiltration happens[115].

The lower mutation burden in PDAC is partially due to poor antigenicity of the tumor microenvironment (TME). The abundant immunosuppressive TME plays a crucial role in blocking T cell activation, which further leads to lower immune therapy efficiency[116]. A significantly increased level of the *TH17* and *Th1* genes acts as an inflammatory immune marker[117]. A variety of immune biomarkers have been reported in PDAC. Those with sound prognostic capability are *CD3, CD4, CD8* and *CD20*. In contrast, increased expression of *CD163, FOXP3, CD204, CD68* and *CD66b* leads to poorer outcomes[114,118].

Interestingly, the presence of T cells has no clinical correlation with PDAC. Tumor-infiltrating CD3+ T cells have been identified in isolated PDAC tumors. However, due to the lack of CD3 zeta expression, the capability to activate T cell-associated receptor signaling has been lost and is under functioning [119]. The tumor immune microenvironment and TME of PDAC is peculiar when compared with other solid tumors because immune therapy does not yield significant results in PanCa treatment. PD-L1, CTLA-4 and PD-1 targeting antibodies are part of immune checkpoints, and inhibition of these targets has been successful in other malignancies but have failed in curative treatment of PanCa[120]. Of note, PD-L1 and PD-1 expression is significantly lower in PDAC than in other tumors. PD-L1 has high expression in almost 30%-40% of PanCa cases, which is correlated with CD8+ cells, thereby indicating a poor prognosis[120,121].

Table 4 Cell-free biomarkers involved in distinguishing pancreatic cancer from other pancreatic benign diseases

Biomarkers	CP	AIP	PanIN	IPMN	MCN
PENK DNA	Yes	No	Yes	Yes	Yes
CUX2	Yes	No	No	No	No
REGIA	Yes	No	No	No	No
TFP12	Yes	No	Yes	Yes	Yes
FOXE1	Yes	No	Yes	Yes	Yes
P16	Yes	No	Yes	Yes	Yes
NPTX2	Yes	No	Yes	Yes	Yes
PENK	Yes	No	Yes	Yes	Yes
CYCLIN D2	Yes	No	Yes	Yes	Yes
CDO1	No	Yes	No	No	No
HAS-mIR-23a	No	No	No	Yes	No
HAS-mIR-23b	No	No	No	Yes	No
HAS-mIR-210	Yes	No	No	No	No
Let-7c	Yes	No	No	No	No
S100A9	Yes	No	No	No	No
BNCI	No	No	Yes	No	No
ADAMTS1	No	No	No	No	No
BNC1	No	No	No	No	No
9-Sep	No	No	No	No	No
GPC1	No	No	No	No	No
PFAA	No	No	No	No	No
OPNT + TIMP-1	No	No	No	No	No
CPA4	No	No	No	No	No
MUC5 AC	No	No	No	No	No
C4BPA	No	No	No	No	No

AIP: Autoimmune pancreatitis; CP: Chronic pancreatitis; IPMN: Intrapapillary mucinous neoplasia; MCN: Mucinous cystic neoplasm; PanCa: Pancreatic cancer; PanIN: Pancreatic intraepithelial neoplasia.

CONCLUSION

Currently, a variety of specimens have emerged as possible sources for liquid biopsy analysis for diagnostic applications. Apart from plasma or serum, urine and sputum have also produced promising disease diagnostic results[44,122]. Liquid biopsies are ideally non-invasive or minimally invasive for sample collection, minimizing patient stress and discomfort. Non-invasive approaches can significantly aid clinical research and utility. Even the recurrence and metastasis of the disease can be potentially monitored based on the cfDNA methods (Table 4).

Therefore, in this review we highlighted the current development in tools for PanCa early diagnosis and prognosis. We demonstrated that useful data can be obtained based on DNA methylation signatures and mutations. The heterogeneity in the pool of cfDNA isolated from a suitable source, such as plasma, contains cfDNA molecules obtained from various origins, and various outcomes, such as DNA methylation or gene mutations, could be obtained from such material. The process of properly understanding the disease source and cfDNA origin has been facilitated significantly by advancements in technologies, such as single cell-based techniques[40]. These advancements can further facilitate liquid biopsy research associated with PanCa biomarker discovery. This also showcases that aberrant DNA methylation signatures harness a significant capability to guide PanCa disease management[32,51]. Approximately 300 clinical trials are currently ongoing that target the use of cfDNA biomarkers in the diagnosis of malignancies. Limitations include cfDNA isolation and sample processing and understanding the biology of ctDNA based on its release from tumors.

The outcome of the secretome can also support PanCa diagnosis significantly. Premalignant lesions such as PanIN are still facing detection challenges because no PanIN-specific protein has been clinically approved. The alterations in cell free nucleic acids does not always manifest into protein changes that can be clinically validated and utilized. However, the secretome in PanCa and secretome biomarkers specific to premalignant lesions are yet to be identified for clinical use but are likely to be highly useful in the future. Validation of biomarkers based on cfDNA alterations and subsequent protein expression can substantially help in the early diagnosis of PanCa and other pancreatic-associated diseases[51,123].

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FOOTNOTES

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MINIREVIEWS

Immunotherapy for recurrent hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is presented frequently in late stages that are not amenable for curative treatment. Even for patients who can undergo resection for curative treatment of HCC, up to 50% recur. For patients who were not exposed to systemic therapy prior to recurrence, recurrence frequently cannot be subjected to curative therapy or local treatments. Such patients have several options of immunotherapy (IO). This includes programmed cell death protein 1 (PD-1) and cytotoxic T- lymphocyte associated protein 4 treatment, combination of PD-1 and vascular endothelial growth factor inhibitor or single agent PD-1 therapy when all other options are deemed inappropriate. There are also investigational therapies in this area that explore either PD-1 and tyrosine kinase inhibitors or a novel agent in addition to PD-1 with vascular endothelial growth factor inhibitors. This mini-review explored IO options for patients with recurrent HCC who were not exposed to systemic therapy at the initial diagnosis. We also discussed potential IO options for patients with recurrent HCC who were exposed to first-line therapy with curative intent at diagnosis.

Key Words: Liver neoplasms; Immune checkpoint blockade; Combination drug therapy; PD-1- PD-L1 blockade; CTLA-4 inhibitor.

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Core Tip: Immunotherapy (IO) has made strong headway in the management of hepatocellular carcinoma (HCC). For patients who recur on local therapy, IO has become the standard of care treatment option for unresectable HCC. The role of IO agents is still not explored in patients who progress on prior IO. This mini-review highlighted the various treatment options available in clinical practice as well as upcoming novel management strategies in recurrent HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with more than 900000 new cases in 2020. HCC accounts for the third most cancer deaths, next only to lung cancer and colorectal cancer. It occurs twice as frequently in males compared to females and is more common in Eastern Asian countries compared to Europe[1]. In the United States (US), there is a shift in the incidence and mortality of HCC from predominantly Asians/Pacific Islanders to African American and Hispanic communities[2]. Such change is most likely due to the successful implementation of hepatitis B virus control measures such as vaccination and effective anti-viral therapy (hepatitis B virus is the main cause of HCC in Eastern Asian populations)[3,4]. On the other hand, nonalcoholic steatohepatitis (NASH) is another common cause of HCC in the Western world and is quickly becoming a key contributor to increasing HCC cases[5]. Between the period of 2010 to 2019, NASH has seen the fastest growth in HCC-associated deaths globally[6]. In the US, NASH is viewed as the most common risk factor (59%), followed by hepatitis C (22%)[7]. Chronic alcohol consumption continues to be a leading cause of HCC as well in the US and other Western countries[8].

Managing patients with early-stage HCC includes local therapy using transplantation, hepatic resection, ablation or transarterial chemoembolization (TACE), but there is always a chance of recurrence. The rate of recurrence was found to be 16% with liver transplantations for HCC, which is the lowest among all local therapy approaches. Thus, for patients eligible for liver transplantation, it is the best treatment option for patients with early HCC[9]. In patients treated with surgical resection, recurrence is seen in > 50% of the patients[10]. Radiofrequency ablation showed recurrence in more than 80% of the patients, either locally or distant at the 5-year follow-up[11]. Surgical resection when compared to ablation for HCC did not show significant improvement in overall survival (OS); however, the disease-free survival period was significantly better for surgical resection[12]. Therefore, resection is often preferred over ablation in HCC. TACE is traditionally used as a bridge to transplantation. For patients who cannot proceed with transplantation, TACE can still provide effective local control. In a large study of 681 patients, of which 287 were treated in the first-line therapy with TACE, recurrence was seen in 43.2% of the patients that achieved complete response (CR)[13].

If HCC recurs, patients can be candidates again for local therapy as described above. However, if they are not amenable to local therapy, systemic therapy is used. There are two types of systemic therapies: (1) Immunotherapy (IO) based; and (2) Non-Immunotherapy based. In this review, we focused on the IO-based systemic approaches.

IMMUNOTHERAPY BASED APPROACHES IN THE FIRST-LINE SETTING

Atezolizumab with bevacizumab

Atezolizumab (Atezo), a programmed death ligand 1 (PD-L1) inhibitor, and bevacizumab (Bev), a vascular endothelial growth factor receptor (VEGF) inhibitor were initially tested in a phase Ib study to evaluate their role for the management of untreated, advanced HCC patients[14-16]. Atezo acts by preventing T cell suppression and selectively inhibiting PD-L1 from attaching to programmed cell death protein 1 (PD-1)receptors[14]. Bev inhibits VEGF, which is commonly associated with progression and development of liver cancer[17]. It acts by inhibiting angiogenesis and tumor growth[18]. The combination of Atezo and Bev can act by reversing VEGF mediated immunosuppression and increased T cell infiltration in the tumor microenvironment, which can be efficacious in treating cancer[19,20].

The IMBRAVE150 study established Atezo in combination with Bev as the standard of care for advanced HCC patients[21] (Table 1). The IMBRAVE 150 (NCT03434379) was a large multicenter, open label phase III randomized study that evaluated the safety and efficacy of Atezo in combination with Bev in comparison to sorafenib in the first-line setting for systemic therapy naïve patients with unresectable HCC[22]. At the time of the first analysis at data cutoff, the OS rate at 12 months (mo) was 67.2% [95% confidence interval (CI): 61.3-73.1] with Atezo + Bev and 54.6% (95%CI: 45.2-64.0) with sorafenib. Median OS (mOS) was not reached for the Atezo + Bev arm and was 13.2 mo (95%CI: 10.4-not reached) for the sorafenib arm. The study had shown median progression-free survival (mPFS) of 6.8 mo (95%CI: 5.7-8.3) for the Atezo + Bev arm and 4.3 mo (95%CI: 4.0 to 5.6) for the sorafenib arm. Thus, the OS and PFS were significantly improved compared to the tyrosine kinase inhibitor (TKI) sorafenib. The Atezo + Bev arm in the study demonstrated a superior overall response rate (ORR) of 27.3% (95%CI: 22.5-32.5) when compared to the sorafenib arm of 11.9% (95%CI: 7.4-18.0), per response evaluation

Table 1 Immunotherapy regimens for first-line use in patients with advanced hepatocellular carcinoma with no prior systemic therapy

Immunotherapy regimen	IMBRAVE150 (NCT03434379)	HIMALAYA (NCT03298451)
Drugs	Atezolizumab, Bevacizumab	Durvalumab, Tremelimumab
Drug class combination	PD-L1 inhibitor, VEGF inhibitor	PD-1 inhibitor, CTLA-4 inhibitor
Study population	Child-Pugh A, ECOG score 0/1, no prior systemic therapy	Child-Pugh A, ECOG score 0/1, BCLC B or C, no prior systemic therapy
Key difference	Portal vein thrombosis patients included	Portal vein thrombosis patients excluded
EGD required?	Yes	No
Overall survival	19.2 mo (95%CI: 17.0-23.7)	16.4 mo (95%CI: 14.2-19.6)
Median progression free survival	6.8 mo (95%CI: 5.7-8.3) *vs* 4.3 (95%CI: 4.0-5.6)	3.8 mo (95%CI: 3.7-5.3)
Overall response rate	27.3% (95%CI: 22.5-32.5)	STRIDE arm: 20.1%
Long-term survival data	Not available	Available

BCLC: Barcelona Clinic Liver Cancer; CI: Confidence interval; CTLA-4: Cytotoxic T lymphocyte associated protein-4; ECOG: Eastern Cooperative Oncology Group; EGD: Esophagogastroduodenoscopy; FDA: Food and Drug Administration; PD-1: Programmed cell death protein-1; PD-L1: Programmed death- ligand 1; STRIDE: Single Tremelimumab Regular Interval Durvalumab; VEGF: Vascular endothelial growth factor.

criteria in solid tumors 1.1 (RECIST 1.1) (P <0.001).

The Atezo+Bev is the only first-line combination regimen involving IO that evaluated high risk patients having Vp4 thrombus, bile duct invasion or liver infiltration > 50%. The improved OS, mPFS and ORR compared to sorafenib regardless of patient etiology and disease risk stamped its role in first-line management of treatment naïve unresectable HCC. The only caveat is that the trial required a pretreatment evaluation of esophageal varices because of its increased complications with cirrhosis and HCC and due to the side effect profile of Bev. Varices, if present, also needed to be treated otherwise the patients were excluded from the trial. Hence, the trial selectively looked at patients who had preserved liver function (Child-Pugh class A) and a decreased risk of variceal bleeding.

At the American Society of Clinical Oncology (ASCO) Gastrointestinal Cancers Symposium 2021, additional data was presented. After a median 15.6mo (range: 0-28.6) follow-up, the mOS was 19.2 mo (95%CI: 17.0-23.7) in the Atezo + Bev arm and 13.4 mo (95%CI: 11.4-16.9) in the sorafenib arm, whereas the mPFS and ORR were similar to the original presented data[23]. The updated data showed 8% of the patients achieving CR with Atezo + Bev compared to < 1% with sorafenib. Moreover, data for a PD-L1 negative patient subgroup did not reveal a meaningful difference in OS, thus suggesting treatment efficacy regardless of PD-L1 expression.

Durvalumab and tremelimumab

Durvalumab (Durva), a PD-L1 inhibitor, and tremelimumab (Treme), a cytotoxic T lymphocyte associated protein 4 (CTLA-4) inhibitor, based on their additive and complementary immunostimulatory activity, were combined in the treatment of HCC[24-26]. At the ASCO 2022 Gastrointestinal Cancers Symposium, the HIMALAYA study was presented. HIMALAYA is an open-label, multicenter, phase III study evaluating the IO combination of Treme+ Durva *vs* sorafenib. Patients with newly diagnosed unresected HCC not amenable to local therapy were initially randomized to the Single Treme Regular Interval Durva (STRIDE regimen) or Durva or sorafenib in a 1:1:1 ratio[27]. The study met the primary endpoint of improved OS in the Treme + Durva arm (STRIDE regimen) when compared to sorafenib. This was also the first study to evaluate long-term OS, with a median follow-up duration of more than 30 mo.

OS was significantly improved for STRIDE *vs* sorafenib [hazard ratio (HR): 0.78; 95%CI: 0.65-0.92; P = 0.0035). The mOS for STRIDE was 16.4 mo (95%CI: 14.1-19.5) *vs*13.7 mo (95%CI: 12.2-16.1) for sorafenib. The mPFS was 3.8 mo (95%CI: 3.7-5.3) in the STRIDE arm and 4.1 mo (95%CI: 3.8-5.5) in the sorafenib arm. Despite a similar PFS for STRIDE and sorafenib, more patients remained progression free at the time of data cutoff for the STRIDE arm. Patients also continued on treatment with STRIDE (46.9%) for at least one cycle compared to sorafenib (36%) past disease progression, which would suggest that more patients derived clinical benefit from this combination. The STRIDE regimen showed superiority in ORR (20.1%) compared to sorafenib (5.1%). In addition, Durva met the objective of OS non-inferiority to sorafenib (HR: 0.86; 95%CI: 0.73-1.03). The ORR was higher for Durva (17.0%) than for sorafenib (5.1%).

In contrast to the IMBRAVE150 study, the HIMALAYA study did not include Vp4 thrombus patients, which is considered a high risk patient group. The subgroup analyses are not available yet[22,27]. The STRIDE regimen was not associated with an increased risk of bleeding with esophageal varices, thus eliminating the need for esophagogastroduodenoscopy for evaluation, as is required for the Atezo+ Bev combination. Therefore, STRIDE can be a very good option for patients who are contraindicated to Bev

(commonly fistula, recent bleeding, high grade varices, severe hypertension and proteinuria).

Even though benefits were seen with the STRIDE regimen, it only involved a single dose of Treme, a CTLA-4 inhibitor, which drives the majority of the toxicities in the IO combination and was seen in this study as well. STRIDE is a proposed treatment regimen for patients who are treatment naïve and have unresectable disease. The treatment was approved for first-line use in October 2022 by the Food and Drug Administration (FDA)[28]. The OS non-inferiority of Durva to sorafenib, along with higher ORR and lower toxicity profile, makes Durva a very attractive option compared to sorafenib. Durva is not FDA approved yet for HCC.

Tislelizumab

The RATIONALE 301 study is a phase III randomized, open label study that evaluated tislelizumab, a PD-1 inhibitor, *vs* sorafenib as first-line treatment for unresectable HCC[29]. The primary objective of the study is to compare OS. The patients have unresectable HCC with no prior systemic therapy, Child-Pugh A class and Eastern Cooperative Oncology Group (ECOG) score 0 or 1. The patients are randomized 1:1 and received either tislelizumab or sorafenib. The study reported non-inferiority of tislelizumab to sorafenib in terms of OS, with a favorable safety profile (mOS: 15.9 mo for tislelizumab *vs* 14.1 mo for sorafenib; stratified HR: 0.85; 95%CI: 0.712-1.019)[30]. Based on the results of this study, single agent tislelizumab can be a potential first-line option for the management of HCC.

Ipilimumab + nivolumab

Checkmate 9DW is another phase III trial evaluating ipilimumab and nivolumab *vs* standard of care TKIs sorafenib or lenvatinib in patients with unresectable HCC who have not received systemic therapy [31]. The primary objective is to measure OS, and the secondary objective is to measure ORR and duration of response.

SRF388

SRF388 is another agent that is being used in combination with Atezo and Bev in the frontline setting for patients with advanced HCC. SRF388 is an inhibitor of interleukin-27 (IL-27), and as a single agent has reduced HCC growth in mouse models[32]. HCC development is suppressed if IL-27 is inhibited in NASH-induced HCC models. Higher levels of IL-27 have also been shown to reduce survival in HCC. IL-27 upregulates PD-L1 expression, lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin-domain containing protein 3 and T cell immunoglobulin and ITIM domain (TIGIT). Thus, combining PD1 therapy with SRF388 increases cytokines such as tumor necrosis factor- alpha and interferon-gamma, which can potentially help in reducing tumor growth.

The preliminary results from a phase I study showed that there were no significant drug-related toxicities (grade > 3 or higher or dose limiting toxicity) and achieved a response similar to preclinical mouse models in humans[33]. Phase II of the study, SRF388-201 study, is currently open and actively recruiting patients who are newly diagnosed with no prior systemic therapy, Child class A, not eligible for TACE and have ECOG 0 or 1. The patients will be randomized 1:1 and will either receive SRF388 or placebo in combination with Atezo and Bev.

CHILD-PUGH SCORE B GROUP

All currently approved therapies are based on studies that exclude Child-Pugh score B patients. There is no prospective data evaluating this group of patients in a first-line setting. A retrospective study evaluated 27 advanced HCC patients with Child-Pugh score B after treatment with Atezo + Bev[34]. The study compared these patients with 130 patients with Child-Pugh score A. Modest activity of the Atezo + Bev combination was seen with an ORR of 14.8% in the Child-Pugh score B group compared to 32.3% for Child-Pugh score A group. mPFS and OS were 3 mo (95%CI: 1.6-4.3) and 6 mo(95%CI: 4.9-7.0), respectively, for Child-Pugh score B compared to mPFS of 6 mo and mOS not reached for Child-Pugh score A group. More grade 3/4 adverse events were observed, with thrombocytopenia and aspartate transaminase elevation being the most common. A higher discontinuation rate was seen in the Child-Pugh score B group.

Similar retrospective studies have also shown that nivolumab and pembrolizumab have a limited role in the management of advanced HCC for Child-Pugh score B/C patients previously treated with other therapies. Poor outcomes were associated with high Child-Pugh score, portal vein thrombosis and diuretic refractory ascites[35,36]. Wong *et al*[36] demonstrated a superior response in Child-Pugh score B7 patients compared to Child-Pughscore B ≥ 8. A trial is currently open that is prospectively evaluating Atezo + Bev combination in HCC patients with Child-Pugh score B7 with no prior systemic therapy[37].

IMMUNOTHERAPY BASED APPROACHES IN SECOND-LINE SETTING

For patients exposed to non-immunotherapeutic agents in first-line

Current strategies involve using immunotherapeutic or non-immunotherapeutic agents in the first-line setting for advanced HCC. For patients who recur following non-immunotherapeutic agents like sorafenib or lenvatinib, several agents are currently approved by the FDA.

Nivolumab +ipilimumab

The Checkmate 040 study was an open label phase I/II dose escalation and expansion trial evaluating single agent nivolumab, a PD-1 inhibitor, in advanced HCC[38] (Table 2). The drug received accelerated approval for use in HCC in patients who progressed on sorafenib. The Checkmate 459 study evaluated nivolumab *vs* sorafenib for HCC. The study did not show significant improvement in OS with single agent nivolumab, which later resulted in the withdrawal of the drug[39,40].

Nivolumab in combination with ipilimumab, a CTLA-4 inhibitor, was also studied in patients with HCC after progression or intolerance to prior therapy in the randomized phase II portion Checkmate 040 study[41]. Majority of the patients received prior sorafenib and included patients who received up to three lines of prior systemic therapy. The study involved three arms with 1:1:1 randomization using different dose combinations of ipilimumab and nivolumab. Arm A had nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (Ipi3 + Nivo1), administered every 3 weeks for four doses, followed by nivolumab 240 mg every 2 weeks. Arm B had nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (Ipi1 + Nivo3), administered every 3 weeks for four doses, followed by nivolumab 240 mg every 2 weeks. Arm C had nivolumab 3 mg/kg every 2 weeks plus ipilimumab 1 mg/kg every 6 weeks.

A total of 148 patients were enrolled. The ORR was 32%, 27% and 29%, respectively, for the three arms. Time to response occurred early and was similar across all treatment arms, regardless of PD-L1 status or baseline alpha-fetoprotein (AFP) levels. The duration of response was also similar. However, mOS were 22.8mo (95%CI: 9.4-not reached), 12.5mo (95%CI: 7.6-16.4) and 12.7mo (95%CI: 7.4-33.0) for Arms A, B and C respectively. Arm A reported higher grade 3/4 treatment-related adverse events (53%) (TRAEs) and higher immune-mediated events compared to Arms B (29%) and C (31%), most likely correlative of the higher dose of ipilimumab. Rash, hepatitis and hypothyroidism were the most common immune-related AEs.

Amongst the three arms, arm A achieved the highest CR rate (8%) with the best OS at 30 mo (44%) and the longest mOS of 22 mo. This treatment of Ipi3 + Nivo1, followed by nivolumab single agent received accelerated approval by the FDA for second-line use in advanced HCC. At the ASCO 2021 Gastrointestinal Cancers Symposium, the 44 mo survival data was presented and continues to show promising results regarding long-term survival and safety profile[42]. A few caveats of the study were that it was an open label phase I/II study without a standard of care control arm and a small number of patients in each arm. The patients were also not stratified per risk factors. However, the study included high risk patients with extrahepatic spread and elevated AFP levels and multiple lines of prior systemic therapy.

Ipilimumab + nivolumab is the standard of care treatment option for patients who progressed or are intolerant to first-line non-immunotherapeutic agents such as sorafenib or lenvatinib.

Pembrolizumab

Keynote 224 is a single arm phase II study of pembrolizumab, a PD-1 inhibitor, in patients with advanced HCC who had progressed on or were intolerant to sorafenib[43]. In total, 104 participants received 200mg of pembrolizumab intravenously every 3 weeks for 2 years or until disease progression, toxicity or withdrawal from the trial. The primary objective of the study was ORR (17%). The mPFS was 4.9 mo (95%CI: 3.4-7.2), and mOS was 12.9 mo (95%CI: 9.7-15.5). TRAEs were observed in 73% of the patients, and 15% of the patients had serious TRAEs. Grade 3/4 TRAEs occurred in about 25% of the patients, with increased alanine transferase, increased aspartate transferase and fatigue being the most common. Immune-mediated grade 3/4 AEs were seen in only 4% of the patients, with adrenal insufficiency being the most common. Based on the data, pembrolizumab is an effective and tolerable option for patients previously treated with sorafenib.

The study also suggested that PD-L1 expression based on combined positive score using tumor and immune cells was correlative of anti PD-1 activity with pembrolizumab. This association was not significant when correlated to tumor positivity score alone. The limitations of the study were that it was a single arm study and did not compare pembrolizumab with a control arm.

Keynote 240 was a phase III global study that tested the efficacy of pembrolizumab with best supportive care (BSC) *vs* placebo with BSC in the second-line setting following progression or intolerance to sorafenib. However, there was no statistical difference seen in mOS or mPFS[44]. The mOS was 13.9 mo (95%CI: 11.6-16.0 mo) for pembrolizumab *vs* 10.6 mo (95%CI: 8.3-13.5 mo) for placebo (HR: 0.78; 95%CI: 0.61-0.99; P = 0.024). mPFS for pembrolizumab was 3.0 mo (95%CI: 2.8-4.1 mo) *vs* 2.8 mo (95%CI: 1.6-3.0 mo) for placebo at the final analysis (HR: 0.72; 95%CI: 0.57-0.90; P = 0.002). The ORR was 18.4%, which was similar to the ORR seen in Keynote 224.

Table 2 Current Food and Drug Administration-approved immunotherapy agents in second-line use post-progression on sorafenib in advanced hepatocellular carcinoma

Immunotherapy agent	Checkmate 040 (NCT01658878)	Keynote 224 (NCT02702414)
Drugs	Ipilimumab, nivolumab	Pembrolizumab
Drug class combination	CTLA-4 inhibitor, PD-1 inhibitor	PD-1 inhibitor
Study population	Child-Pugh A, ECOG score 0/1, prior systemic therapy with sorafenib or intolerance to sorafenib	Child-Pugh A, ECOG score 0/1, prior systemic therapy with sorafenib or intolerance to sorafenib
Overall survival	22.8mo (95%CI: 9.4-not reached)	12.9 mo (95%CI: 9.7-15.5)
Median progression free survival	3.9 mo (95%CI: 2.6-8.3)	4.9 mo (95%CI: 3.4-7.2)
Overall response rate	32%	18%
Most common treatment related AE	Rash, hepatitis, hypothyroidism	Hypothyroidism, hepatitis, adrenal insufficiency
Child-Pugh score B group studied	No data available	Retrospective data available
FDA approval	Yes	Yes

CI: Confidence interval; CTLA-4: Cytotoxic T lymphocyte associated protein-4; ECOG: Eastern cooperative oncology group; FDA: Food and drug administration; PD-1: Programmed cell death protein- 1.

Keynote 394 is another phase III randomized study evaluating pembrolizumab + BSC *vs* placebo + BSC, specifically in Asian patients with advanced HCC with progression on or intolerance to sorafenib or oxaliplatin chemotherapy. Early results were presented at ASCO 2022, and they showed that pembrolizumab with BSC improves OS, PFS and ORR in Asian patients[44]. At the final analysis, pembrolizumab significantly improved mOS *vs* placebo. mOS was noted to be 14.6 mo (95%CI: 12.6-18.0) for pembrolizumab *vs* 13.0 mo (95%CI: 10.5-15.1) for placebo. According to the protocol, if OS was superior, PFS and ORR at the second interim analysis were studied. Pembrolizumab significantly improved PFS (HR: 0.74; 95%CI: 0.6-0.9; P = 0.003) and ORR (estimated difference: 11.4%; 95%CI: 6.7-16.0; P = 0.00004).

Based on these studies, PD-1 single agent may have a differential benefit according to various pharmacodynamic changes amongst ethnic groups. Pembrolizumab therefore could be a better tolerated option for patients with progression or intolerance to first-line non-IO based agents, particularly in Asian patients.

For patients exposed to immunotherapeutic agents in first-line

There is no prospective data for any therapy in patients who recur following first-line IO. Clinical trials are currently underway exploring this space.

Wong *et al*[46] performed a retrospective analysis of 25 patients who had previously progressed on prior immune checkpoint inhibitor (ICI) monotherapy or combined therapy. Patients received ipilimumab in combination with either nivolumab or pembrolizumab. The 3-year follow-up data revealed that ORR was 16%, and CR rate was 12%. Overall, 40% of the patients achieved clinical benefit with this regimen, with a median duration of response of 11.5 mo (2.7-30.3 mo), and mOS were 10.9 mo. The drugs had an acceptable safety profile.

In clinical practice, when patients desire second-line IO after progression on first-line IO, we can potentially use agents that have not been tried in the first-line setting. Treme and Durva, which is an IO+IO combination can be tried after progression on Atezo and Bev, which is an IO + VEGF combination. The reverse order can also be offered for patients who are offered IO+ IO combination first. Further clinical trials in this space are also required to evaluate the role of these agents post-progression.

COMBINATION THERAPY TRIALS WITH SYSTEMIC THERAPY

Several non-immunotherapeutic agents have been approved by the FDA for use in the management of advanced HCC, either in first-line or second-line settings post-progression. Trials are ongoing in this space to evaluate their potential role in combination with an immunotherapeutic agent (Table 3).

Camrelizumab, an anti PD-1 inhibitor, in combination with rivoceranib, an anti-VEGF receptor type 2 TKI (apatinib), is the first phase III study to show positive survival benefits with a PD-1/PD-L1 inhibitor and anti-angiogenic TKI for unresectable HCC[47]. In this randomized, open-label, phase III trial, 543

Table 3 Possible treatment regimens for patients with advanced hepatocellular carcinoma who have recurred on local therapy

Patient group	Treatment	Status
Advanced HCC patients with no prior systemic therapy	Atezolizumab + bevacizumab	FDA approved for first-line use (no contraindications to atezolizumab/bevacizumab or both)
	Durvalumab + tremelimumab	Contraindications to atezolizumab or bevacizumab or both; FDA approved for first-line use
	Single agent immunotherapy	Poor ECOG 3-4
Advanced HCC with prior systemic therapy with TKIs like sorafenib or lenvatinib	Ipilimumab + nivolumab	FDA approved for second-line use
	Pembrolizumab	FDA approved for second-line use; High risk subgroups: Asian patients, poor ECOG
	Atezolizumab + bevacizumab	Warrants further evaluation
	Durvalumab + tremelimumab	
	Clinical trials	
HCC patients with prior IO based systemic therapy	Partner switching from currently available first-line options	Using drugs with different mechanism of action in comparison to first line IO therapy
	Pembrolizumab	Warrants further evaluation
	Ipilimumab + nivolumab	
	Clinical trials	

ECOG: Eastern cooperative oncology group; FDA: Food and drug administration; HCC: Hepatocellular carcinoma; IO: Immunotherapy; TKI: Tyrosine kinase inhibitor.

patients were randomized 1:1 to receive camrelizumab (C) + rivoceranib (R) /apatinib or sorafenib. Patients were stratified by macrovascular invasion and/or extrahepatic metastases, geographical region (Asia *vs* non-Asia) and baseline serum AFP (< 400 *vs* ≥ 400 ng/mL). The primary endpoints were PFS as well as OS. With a median follow-up time of 7.8 mo, PFS was significantly improved with C + R *vs* sorafenib [median 5.6 mo (95%CI: 5.5-6.3) *vs* 3.7 mo(2.8-3.7); HR: 0.52;95%CI: 0.41-0.65; $P < 0.0001$]. With a median follow-up of 14.5 mo, OS was significantly prolonged with C + R *vs* sorafenib [median 22.1 mo (95%CI: 19.1-27.2) *vs* 15.2 mo (13.0-18.5); HR: 0.62; 95%CI: 0.49-0.80; 1-sided $P < 0.0001$]. ORR, disease control rate and duration of response were also better with C+R *vs* sorafenib. Grade ≥3 TRAEs occurred in 80.9% with C + R and 52.4% with sorafenib. TRAE led to discontinuation of any treatment in 24.3% (of both agents in 3.7%) with C + R and 4.5% with sorafenib.

Keynote 524 was a phase Ib study to assess the antitumor activity of lenvatinib in combination with pembrolizumab. The initial data showed that the combination was safe for use with no drug limiting toxicities, and Grade ≥ 3 toxicities were seen in 67% of the patients. The ORR was 36% per RECIST 1.1, with 1 patient having CR. Median duration of response was 12.6 mo, and the ORR findings were consistent for subgroups with poor prognostic features. The time to treatment response was less than 2.0 mo, with mPFS of 8.6 mo and mOS of 22.0 mo[48].

Based on this promising activity, a phase III study, LEAP-002, tested pembrolizumab + lenvatinib as a combination therapy[49]. Patients (n = 794) were randomized 1:1 for lenvatinib + pembrolizumab *vs* lenvatinib + placebo. The dual primary endpoints of the study were OS and PFS. After the follow-up, the authors observed 17.6 mo for the final PFS and 32.1 mo for the final OS. The primary endpoints of OS and PFS did not meet pre-specified statistical significance. The mOS with lenvatinib + pembrolizumab was 21.2 mo *vs* 19.0 mo with lenvatinib, and the HR was 0.840 (95%CI: 0.708-0.997; $P = 0.0227$). mPFS at final analysis was 8.2 mo for lenvatinib + pembrolizumab *vs* 8 mo for the lenvatinib alone arm. HR for PFS at interim analysis 1 was 0.867 (95%CI: 0.734-1.024; $P = 0.04660$. ORR at final analysis was 26.1% for lenvatinib + pembrolizumab *vs* 17.5% for lenvatinib. Grade 3-5 TRAEs were 62.5% in the lenvatinib + pembrolizumab arm and 57.5% in the lenvatinib arm (grade 5). Notably, in the LEAP-002 trial, OS with lenvatinib monotherapy was the longest observed with a TKI (19.0 mo), which was much longer than the mOS of Lenvatinib (13.6 mo) shown in the REFLECT trial[50]. Based on the data, a meaningful difference in activity was not seen with lenvatinib + pembrolizumab *vs* lenvatinib monotherapy alone.

Cosmic 312 is a phase III trial comparing cabozantinib plus Atezo *vs* sorafenib as first-line systemic treatment for advanced HCC[51]. Patients with tumors invading the main portal vein were not excluded from the trial. Patients were randomly assigned (2:1:1) to cabozantinib 40 mg orally once daily plus Atezo 1200 mg q3 weeks, sorafenib 400 mg orally BID or single agent cabozantinib 60 mg orally once daily. Primary endpoints for the study were PFS in the first 372 patients in the intention to treat patient population and OS for all patients. mPFS was 6.8 mo (95%CI: 5.6-8.3) in the combination treatment

group *vs* 4.2 mo (95%CI: 2.8-7.0) in the sorafenib group (HR: 0.63; 95%CI: 0.44-0.91; $P = 0.0012$). mOS (interim analysis) was 15.4 mo (95%CI: 13.7-17.7) in the combination treatment group *vs* 15.5 mo (12.1-not estimable) in the sorafenib group (HR: 0.90; 95%CI: 0.69-1.18; $P = 0.44$).

NOVEL AGENTS

Several novel IO-based agents are currently in development that could have a potential role in the management of HCC.

LAG-3 inhibitors are potential agents in development and are currently being tested in their role in HCC. LAG-3 inhibition leads to the activation of exhausted T cells. Relatlimab, a LAG-3 inhibitor, is currently being tested with nivolumab for potential use in patients who have progressed on first-line TKIs, like sorafenib, and are IO naïve[52]. The agent is also being investigated in combination with nivolumab and Bev in treatment naïve unresectable HCC patients[53].

A novel therapy targeting glypican-3 (GPC-3) using chimeric antigen receptor T cells is underway in advanced HCC. Early results from two phase I studies have demonstrated their safety, with 2 patients out of 13 showing partial response[54]. Glypican-3 expression has been associated with a worse prognosis in HCC[55]. There are several trials underway in this space. Natural killer cell activity has also been potentially linked to an increased risk of recurrence following curative treatment of HCC[56]. FT500 and FATE NK-100 are some of the natural killer cell IO trials currently in development for their potential role in HCC[57,58].

CONCLUSION

The scope of IO in the management of HCC is indeed promising. We have moved beyond sorafenib, the standard of care in the first-line management of advanced HCC for the past decade[59]. Atezo in combination with Bev, based on the IMBRAVE150 study, can now be considered the new standard of care for patients who have a recurrence of disease and are not amenable to local therapy. The STRIDE regimen, based on the HIMALAYA study, can also be considered a potential option if a patient is not a good candidate for the IMBRAVE regimen. For patients previously treated with sorafenib and recur or progress, ipilimumab + nivolumab or pembrolizumab are currently identified agents in the second-line setting. In their study, Wong *et al*[46] have shown that continuing to use IO agents in the second-line setting post-progression on prior ICI is certainly protective. Clinical trials to evaluate the role of ICIs in this space are undoubtedly necessary. Partner switching such as using PD-1/PD-L1 inhibitors, VEGF-inhibitors or CTLA-4 inhibitors based on the currently approved therapies should also be evaluated in the second-line setting. The role of these agents in patients with Child-Pugh score B also needs further evaluation. We are also looking at emerging combinations of non-immunotherapeutic agents like lenvatinib and cabozantinib with immunotherapeutic agents, based on the LEAP-002 and COSMIC-312 trials. Further clinical trials are warranted to assess these agents' roles in managing HCC.

With the increasing use of immunotherapeutic agents in the neoadjuvant and adjuvant setting for early-stage HCC, we will see patients exposed to IO agents before recurrence and require systemic therapy. These patients may recur while still being on treatment with an IO agent or can recur after completion of treatment. The scope of immunotherapeutic agents in this setting will further need exploration. There is an unmet need for clinical trials to evaluate treatments involving HCC. Further immunotherapeutic agents are already being developed to improve the existing agents in the first-line setting.

FOOTNOTES

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MINIREVIEWS

Intestinal ultrasound as a non-invasive tool to monitor inflammatory bowel disease activity and guide clinical decision making

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Abstract

Intestinal ultrasound (IUS) is a non-invasive, real-time, cross-sectional imaging tool that can be used at the point-of-care to assess disease activity in patients with Crohn's disease or ulcerative colitis. IUS promotes quick and impactful treatment decisions that can modify disease progression and enhance patient compliance. This review will summarize the technical aspects of IUS, the evidence to support the use of IUS in disease activity monitoring, the comparison of IUS to current standard of care monitoring modalities such as colonoscopy and calprotectin, and the optimal positioning of IUS in a tight-control monitoring strategy.

Key Words: Crohn's disease; Ulcerative colitis; Disease monitoring; Intestinal ultrasound

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Core Tip: Intestinal ultrasound (IUS) is a non-invasive, real-time, cross-sectional imaging tool that is currently underutilized for direct disease activity monitoring in Crohn's disease (CD) and ulcerative colitis (UC). IUS is the optimal point-of-care method to monitor disease activity in patients with CD or UC with excellent patient compliance and comparison to such traditional monitoring modalities as calprotectin, colonoscopy, and magnetic resonance enterography.

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INTRODUCTION

The current conventional approach to inflammatory bowel disease (IBD) monitoring in the United States includes routine patient visits to the gastroenterology clinic every 3-6 mo with more urgent or frequent visits based on symptomatology. During these visits, an assessment of the patient's current disease status is undertaken *via* history and physical examination. After the visit, the evaluation typically continues with biomarkers such as C-reactive protein and fecal calprotectin (FC), colonoscopy, and magnetic resonance enterography (MRE) though results are often delayed and compliance rates low. This traditional, fragmented model of IBD monitoring is not well suited to detect subclinical inflammation or prevent disease progression, and has contributed to a therapeutic effectiveness ceiling of 30%[1].

Intestinal ultrasound (IUS) is a non-invasive, real-time, cross-sectional imaging tool that is currently underutilized for direct disease activity monitoring in Crohn's disease (CD) and ulcerative colitis (UC)[2]. Use of IUS at the point-of-care in a tight monitoring model can add significant value to the routine clinic visit by allowing the treating gastroenterologist to accurately and immediately assess disease activity and treatment responsiveness, something that is sorely lacking in the current traditional IBD care model. Furthermore, IUS allows for the easy assessment of transmural healing (TH), a target beyond simple mucosal healing (MH) that may impact long-term disease outcomes.

In this review, we discuss key concepts to understand how IUS utilization will impact care delivery for patients with IBD and achieve disease modification. We will review the technical aspects of IUS, evidence for detection of disease activity and treatment responsiveness, comparison to current standard of care management tools such as MRE, FC, and ileo colonoscopy, impact on shared understanding and real-time decision making, optimal utilization, and future applications.

TECHNICAL ASPECTS OF IUS

Basic ultrasound machine requirements

Use of IUS as a cross-sectional tool for disease activity monitoring starts with a minimum requirement of a modern ultrasound machine available for regular use. Two probes are needed with ability to assess color Doppler signal (CDS) - a lower-frequency convex array probe (3-5 MHz) for global evaluation of the entire bowel and assessment of complications with ability for deeper penetration, and a higher-frequency linear array probe (5-15 MHz) for visualization of the five bowel wall layers and measurement of bowel wall thickness (BWT) to the level of 0.1 millimeters (mm)[3].

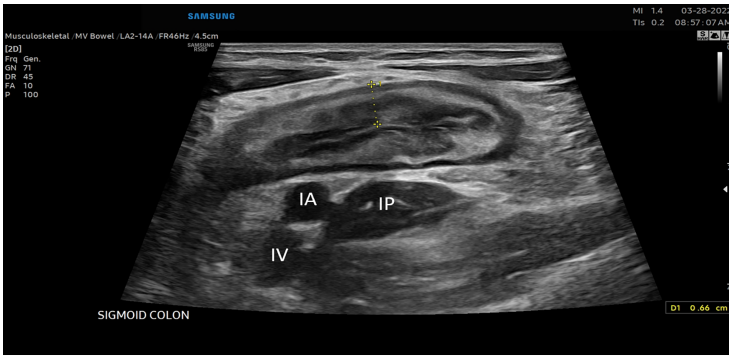
Patient preparation and the role of contrast

Fasting or bowel preparation prior to IUS is unnecessary for optimal visualization of the bowel and the examination can be performed without any prior planning. Intravenous [Contrast Enhanced Ultrasound (CEUS)] or oral contrast [Small Intestinal Contrast Ultrasound (SICUS)] are not beneficial during point-of-care evaluation in IBD activity monitoring and treatment response assessment. The value of CEUS and SICUS, based on local availability and expertise, is in the assessment of suspected complications. CEUS is a valuable tool in the detection of and differentiation of phlegmon and abscesses compared to traditional non-contrast IUS[4]. SICUS adds further value over IUS in the evaluation of proximal small bowel (proximal ileal and jejunal) lesions and detection of stenoses, but the ingestion of oral contrast leads to extended time for completion of the examination that is no longer suitable for use as a point-of-care assessment tool[5,6].

IUS examination

IUS examination should begin with an initial assessment of the distal sigmoid colon using the convex probe beginning in the left lower quadrant of the abdomen or left suprapubic region with identification of the iliac vessels and left iliopsoas muscle, fanning the probe up with the use of graded compression to identify air within the lumen of the distal sigmoid colon and visualization of the subsequent bowel wall layers (Figure 1).

The colon should then be tracked proximally visualizing the retroperitoneal descending colon to the left inferior border of the intercostals or just below to denote the splenic flexure, then to identify the transverse colon utilizing the thick wall of the stomach as an anatomical landmark, then to the hepatic flexure, downward toward the right lateral hemiabdomen to visualize the ascending colon, cecum, and



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Figure 1 Inflamed sigmoid colon located super to the iliac vessels and iliopsoas muscle in the left lower quadrant of the abdomen. IA: Iliac artery; IV: Iliac vein; IP: Iliopsoas muscle.

ultimately to the right lower quadrant of the abdomen and/or right suprapubic region to identify the ileocecal valve, and terminal ileum, again using the right lower quadrant iliac vessels and iliopsoas muscle as a landmark for proper identification of the terminal ileum superior. The remainder of the proximal small intestine should be scanned in a "lawn-mower" technique, sweeping up and down over the mid-abdomen, to visualize the remaining proximal small bowel. Differentiation of the large intestine and small intestine from one another during this process is essential and done easily based on the hypermotility and movement of intestinal contents within the small bowel compared to that of the colon. Once the global initial assessment of the bowel is complete with a convex probe, careful repeat assessment of the entire colon and small bowel should be performed for IBD activity assessment with the linear high-frequency probe. Measurements for BWT should be performed for each segment in both longitudinal and transverse planes, with an average of measurements taken at least 1 cm apart denoting the thickness between the lumen-mucosal interface and the muscularis-serosal interface (Figure 2). CDS assessment of the bowel wall for hyperemia should be performed in each segment with use of the modified Limberg score (Figure 3). The other two parameters that should be assessed in each segment are inflammatory fat (iFat) and the presence or loss of bowel wall layer stratification.

Bowel segments for IUS activity monitoring

The primary bowel segments assessed during every IUS examination are the sigmoid colon, descending colon, transverse colon, ascending colon, cecum, and terminal ileum[7]. Visualization of the rectum can be challenging and typically only performed with adequate resolution for disease activity assessment in patients with lower body mass indices and full bladders at the time of examination. Even with visualization of the rectum, normal values are not well defined, thus making it even harder for use at the point-of-care for disease activity assessment without benchmark with another non-invasive biomarker such as FC or a recent endoscopic evaluation. Additionally, the proximal small bowel beyond 30 cm of the ileum remains challenging and even an expert utilizing the proper technique may miss proximal ileal or jejunal inflammation relying on evaluation with IUS alone (Table 1).

MONITORING DISEASE ACTIVITY AND TREATMENT RESPONSE

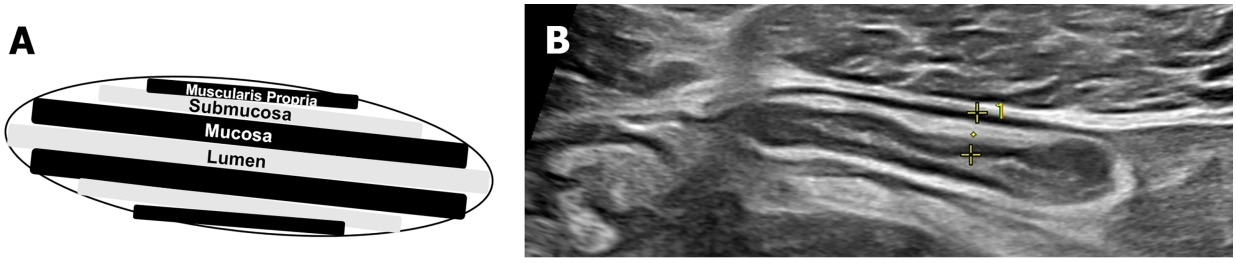
Features of disease activity

IUS assessment of BWT is the primary and most important measure of disease activity in both CD and UC and has been shown to correlate with endoscopic inflammation[8]. In adults, a BWT cut-off > 3 mm in any segment is the most consistent individual IUS parameter that correlates with active endoscopic disease, with a sensitivity and specificity of 88%-89% and 93%-96% respectively[9,10]. When assessing the colonic bowel segments in patients with IBD, the per patient sensitivity and specificity of IUS to detect disease activity is 90% and 96% respectively[11]. UC is a transmural disease and thus IUS is an excellent option to monitor disease activity non-invasively and assess disease extension in patients with ulcerative proctitis[12]. In a retrospective study of patients with UC, BWT alone was shown to correlate with the Mayo endoscopic score[13]. In children, there is no agreed upon cut-off for BWT that correlates with endoscopic activity, however a BWT < 2 mm is almost always normal[14].

After BWT, hyperemia assessed by CDS is the next most important measure of disease activity. Assessment of CDS alone has been shown to correlate with CD clinical activity[15-17]. When combined with increased BWT, increased hyperemia is almost always representative of active disease. In fact, in development of both CD and UC IUS scores with endoscopy, multivariable analysis demonstrates that often the only independent predictors of endoscopic activity are BWT and hyperemia[18-20]. Additional

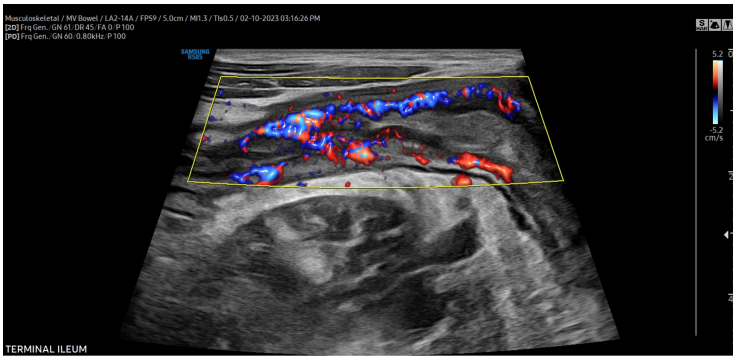
Table 1 Optimal and limited bowel segments for intestinal ultrasound disease activity monitoring

Optimal bowel segments	Limited bowel segments
Sigmoid colon	Rectum
Descending colon	Splenic flexure
Transverse colon	Proximal ileum
Ascending colon	Jejunum
Ileocecal valve	
Terminal ileum	



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Figure 2 Measurements for bowel wall thickness. A: Schematic of bowel wall layers; B: Bowel wall thickness measurement in the inflamed terminal ileum, yellow lines indicate the measurement of bowel wall thickness from the lumen-mucosa interface to the muscularis propria-serosal interact.



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Figure 3 Severe hyperemia assessed by color Doppler signal. Color Doppler signal graded by the modified Limberg score. Shown here is a modified Limberg score of 3 in the terminal ileum. A score of 0 = absent signal. A score of 1: Short signals inside the bowel. A score of 2: Long signals inside the bowel, and a score of 3: Long signals inside and outside of the bowel.

characteristics of inflammation in IBD are focal disruption of the bowel wall layers, and hypoechoic or hyperechoic changes in the layers representing a loss of bowel wall stratification. Inflammatory mesenteric fat wrapping is seen, appearing hyperechoic encasing the inflamed bowel.

IUS activity scores

There have been more than twenty IUS activity scores developed for adults with CD and UC, with the most common and important parameters being BWT, followed by both hyperemia and bowel wall stratification[21]. Only a few recently developed IUS scores have been validated with a standardized endoscopic score. They are simple and can be used at the point-of-care for clinical decision making without significant time for calculation (Table 2)[19,20,22-24]. Only two scores specific to children with IBD have been developed, one CD and one UC, and only the UC score is validated with an endoscopic score[24,25]. A novel IUS score recently developed by a group of international experts, the International Bowel Ultrasound Group (IBUS)-SAS (score 0-100), is yet to be validated and is more complex. Although its clinical utility may be limited, it may serve as a novel score for disease monitoring and treatment response in clinical trials[26].

Table 2 Interpretation of validated and simple intestinal ultrasound activity scores for Crohn's disease and ulcerative colitis

IUS scoring system	BWT (mm)	iFAT	Modified LS	LBWS	Other
Crohn's disease indices					
SUS-CD (0-5)[19]	0: < 3.0 1: 3.0-4.9 2: 5.0-7.9 3: ≥ 8.0		0: LS 0 1: LS 1-2 2: LS 3		
Simple US score[20]	0.957 (BWT)		0.859 (LS)		
Ulcerative colitis indices					
MUC[21,24]	1.4 (BWT)		0: LS 0 2: LS 1-3		
UC-IUS (0-7)[25]	0: ≤ 2 1: 2.1-3.0 2: 3.1-4.0 3: > 4	0: Absent 1: Present	0: LS 0 1: LS 1 2: LS 2-3	0: No loss 1: Loss is present	
Civitelli UC index (0-4)[26] (pediatric)	0: ≤ 3 1: > 3		0: LS 0 2: LS 1-3	0: No loss 1: Loss is present	Abnormal haustrations (0: Normal, 1: Abnormal)

IUS: Intestinal ultrasound; BWT: Bowel wall thickness; iFat: Inflammatory fat; LS: Limberg Score; LBWS: Loss of bowel wall stratification; UC: Ulcerative colitis; CD: Crohn's disease.

Monitoring treatment response in CD and UC

IUS is a reliable tool to monitor treatment response in both CD and UC. Improvement in all IUS parameters (BWT, hyperemia, iFat, bowel wall stratification) can be seen within 12 wk of treatment initiation, with faster changes seen in certain patients and in the colon as compared to the ileum. (30) How to interpret these changes, including defining transmural remission, and the timing of repeat IUS assessments remains an evolving concept.

In patients with CD prescribed anti-inflammatory IBD therapy, a large multicenter German study demonstrated that improvement in all IUS measures can be seen at both 3 and 12 mo[27]. TH, defined as a BWT ≤ 3 mm and absent hyperemia by CDS, has been shown to be achievable in approximately 25%-31% of adult patients with CD after 2 years of treatment[28-32]. The STARDUST sub-study recently evaluated the effect of ustekinumab on TH monitored by IUS and demonstrated increasing TH rates to a total of 24.1% by week 48[33]. In 40 adult patients with endoscopically active CD initiating anti-tumor necrosis factor therapy, an 18% decrease in BWT 4-8 wk post-induction predicted endoscopic response [area under the receiver operating characteristic curve (AUROC) =0.77, odds ratio (OR) = 10.80, P = 0.012] and a BWT cut-off of 3.2 mm was accurate to detect endoscopic remission (AUROC = 0.94, OR = 39.42, P < 0.0001) at weeks 12-32[34]. Lastly, a recent multicenter Italian study of adult patients with CD treated with various biologic therapies demonstrated higher rates of TH (defined as normalization of all IUS parameters) with improvement in IUS in 53% at 3 mo and 64% by 1 year with a number needed to treat of 3.6 patients in order to achieve TH. Compared to MH alone, TH is associated with improved outcomes and a decreased risk of long-term disease progression[29,30,35,36].

Similarly, there is evidence in patients with UC to support the use of IUS as a monitoring tool of treatment response, and in fact, changes on IUS may be seen quicker in patients with UC compared to those with ileal CD. Evidence comes from a large multicenter German study of patients with UC who experienced clinical relapse and were treated with anti-inflammatory therapies; there was improvement in the colon BWT as early as 2 wk and BWT improvement continued 12 wk after treatment initiation [37]. In a study of 74 patients with UC, those who did not have a significant treatment response on IUS by 3 mo, measured by a significantly increased BWT to > 6 mm and presence of hyperemia, were at increased risk of continued severe endoscopic activity at 15 mo[38]. In 27 patients with UC treated with tofacitinib, BWT correlated with Mayo score (r = 0.68, P < 0.0001) and UC Endoscopic Index for Severity (r = 0.73, P < 0.0001) at induction and week 8 after treatment initiation. A decrease in BWT was more pronounced in patients with endoscopic response with a decrease in BWT of 32% from baseline being accurate for endoscopic response (AUROC = 0.87) and a BWT cut-off of 2.8 mm being the most accurate value to detect endoscopic remission (AUROC = 0.87)[39]. Utilizing IUS at baseline in patients with UC can also predict disease course. A Milan Ultrasound Criteria score > 6.2 at baseline in a study of 98

patients with UC was predictive of a negative disease course (need for corticosteroids, change in therapy, hospitalization, or colectomy) at 1.6 years after treatment initiation[40].

Utility for monitoring in pregnancy

While it is critical to control IBD activity during pregnancy, the tools for tight control disease activity monitoring are limited. IUS provides significant value for the precise monitoring of disease activity in the colon throughout all three trimesters and in the terminal ileum during the first two trimesters. In an Australian cohort of 90 pregnant women with IBD, 127 IUS examinations were performed. Adequate ileal views for disease activity assessment were obtained in 93% of patients at less than 20 wk gestational age, but in only 56% of patients at 20-26 wk. In contrast, adequate colonic views were obtained in 91% of all IUS examinations[41]. In this cohort, BWT was compared to FC as the current reference standard for disease activity monitoring during pregnancy and BWT was found to positively correlate with FC (r = 0.26, P = 0.03) and had a sensitivity of 74%, specificity of 83% and negative predictive value of 90%. Similarly, in a small Dutch cohort of 38 pregnant women with IBD, 27 of whom were followed with serial IUS examinations during pregnancy, feasibility to assess the terminal ileum significantly decreased from 91.3% in the first trimester to 21.7% in the third trimester (P < 0.0001). When compared to FC and clinical activity combined, IUS was able to distinguish active from quiescent disease with 84% sensitivity and 98% specificity[42].

Utility for monitoring in pediatrics

In children, use of IUS as a non-invasive tool to monitor disease activity is of even more importance than in adults, as younger children often require sedation for MRE and there is a need to reduce the risk of radiation exposure from repeated CT scans. As previously stated, there is a clear consensus that a cut-off value for normal BWT in children is not 3 mm, but more likely to be in the range of 2-2.5 mm[14]. Similar to adults, IUS can be used to monitor treatment response in children with CD treated with infliximab. In a study of 28 children with newly diagnosed ileal CD, BWT, hyperemia and involved segment length all significantly decreased as early as 2 wk after infliximab initiation and there was a strong correlation between CDS and FC (r = 0.71, P < 0.0001). Linear mixed models from this study demonstrated that BWT, hyperemia, and involved segment length continue to decrease over the course of 6 mo after infliximab initiation[43]. In a small pilot study of 13 children with small bowel CD undergoing infliximab induction, bowel wall hyperemia decreased in all but one patient post-induction (P = 0.01), indicating that hyperemia assessed by CDS may be the earliest IUS measure to normalize post-induction[44].

Utility for monitoring postoperative CD recurrence

IUS is an accurate tool for monitoring postoperative CD recurrence in the neo-terminal ileum after ileocolic resection. BWT greater than 3-3.5 mm is accurate to detect recurrence based on ileocolonoscopy with a sensitivity of 90%-100%[45]. In a study using both traditional IUS and CEUS to assess CD recurrence, 90 patients, 62 of which had severe recurrence (Rutgeerts score i3 or i4), underwent IUS, CEUS, and endoscopy. A BWT > 5 mm, without any additional parameters, demonstrated 100% specificity to detect recurrence and a BWT > 6 mm was 95.7% specific to detect severe recurrence. The addition of bowel wall contrast enhancement ≥ 70% to either BWT ≥ 5 or 6 mm was the most accurate to the Rutgeerts score with an AUROC of 0.89 for detecting recurrence[46].

COMPARISON WITH OTHER CROSS-SECTIONAL IMAGING MODALITIES

Overall IUS, MRE, and CTE have similar accuracy for the diagnosis of CD complications such as strictures, abscesses, and fistulae[47]. In the largest study to date, the prospective multi-center METRIC trial included 284 patients, 233 of which had small bowel CD, and demonstrated that IUS and MRE are comparable for detection of disease in the terminal ileum. IUS had a sensitivity and specificity of 92% and 84% while MRE had a sensitivity and specificity of 97% and 96%. MRE was superior to IUS for the detection of small bowel disease extent with a sensitivity of 80% compared to 70% for IUS. Similarly, in a multicenter Italian study of 234 adult patients with CD, IUS and MRE had comparable accuracy with a sensitivity and specificity to detect inflammation of 96% and 97% for IUS and 96% and 94% for MRE. MRE was more accurate than IUS to define small bowel disease extension (r = 0.69) and detect fistulae (k = 0.67), but comparable for detection of strictures (k = 0.82) and abscesses (k = 0.88)[48]. As such, the updated guidelines from the European Crohn's and Colitis Organization (ECCO)-European Society for Gastrointestinal and Abdominal Radiology and ECCO-European Society for Pediatric Gastroenterology, Hepatology, and Nutrition now propose the use of IUS in the diagnostic evaluation for adult and pediatric CD[49,50].

Follow-up analysis from the METRIC trial for observer agreement demonstrated that IUS and MRE are similar, with IUS performing slightly better than MRE numerically. Interobserver agreement for MRE was modest for new diagnosis [68% (k = 0.36)] and relapsed patients [78% (k = 0.56)] and only slight for colonic assessment for new diagnosis [61% (k = 0.21)] and relapsed patients [60% (k = 0.20)]

[51]. Interobserver agreement for IUS was higher than MRE in the small bowel for new diagnosis [82% (k = 0.64)] and for relapsed patients [81% (k = 0.63)] and in the colon for new diagnosis [64% (k = 0.27)] and relapsed patients [78% (k = 0.56)][52]. Furthermore, a retrospective study in children with IBD demonstrated that MRE is not accurate for the assessment of colonic disease, with the simplified Magnetic Resonance Index of Activity unable to identify severe lesions in colonic segments[53].

IMPACT ON PATIENT MANAGEMENT AND SHARED DECISION MAKING

When determining the optimal tight control monitoring tool, perhaps the most important aspect to consider is patient preference and compliance. IUS is one of the most preferred monitoring tool by patients with IBD. In a survey of 121 Australian patients with a formal diagnosis of IBD, IUS scored highly acceptable for disease activity monitoring (mean 9.20 ± 1.37) compared to colonoscopy (7.94 ± 2.30), FC (8.17 ± 1.96), serum sampling (8.87 ± 1.62) and alternative forms of cross-sectional imaging (8.67 ± 1.60)[54].

Beyond preference, use of IUS for IBD monitoring enhances shared understanding and increases the ability of providers to make major treatment decisions during routine clinic visits. In a study of patients randomized to ultrasound-driven IBD care *vs* non-ultrasound driven IBD care, patients who underwent IUS reported better understanding of all aspects of their disease and symptoms, and were more confident in their ability to make informed decisions about managing their disease[55]. Furthermore, gastroenterologists altered management by changing medications in 47% of patients in the ultrasound group compared to just 22% in the non-ultrasound group (P = 0.002). Based on disease activity, providers were more likely to change therapy when patients had an IUS compared to when they did not (P = 0.024).

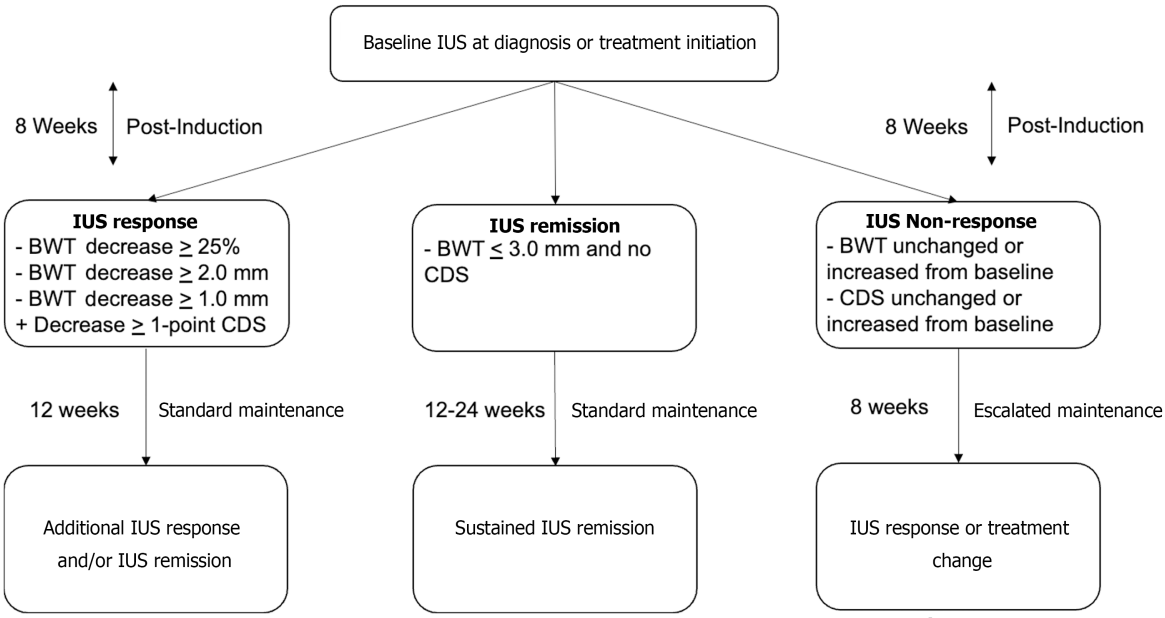
POSITIONING ALGORITHM AT THE POINT OF CARE IN THE CLINIC

Debate over the timing and utilization of IUS for tight control monitoring, as well as the definition of treatment response and remission, is ongoing. Multiple studies demonstrate a decrease in BWT by week 4 after therapy initiation, but the longitudinal effect of response reassessment at this early timepoint is still unclear[43,56]. A panel of gastroenterologists from the IBUS developed an expert consensus statement and defined: (1) IUS response as a decrease in BWT by 25% from baseline or 2.0 mm, or greater than 1.0 mm with a decrease in CDS by the modified Limberg score of one grade; and (2) IUS remission as a BWT ≤ 3 mm and normal CDS or absence of hyperemia[57].

Here we propose a practical tight control monitoring algorithm based on IUS (Figure 4). IUS should be repeated post-induction at week eight, regardless of biologic therapy, to assess change in BWT and hyperemia by CDS. If there is no change or an increase in BWT and/or hyperemia, optimization of therapy (dose or interval escalation) should be considered. Repeat IUS should then occur again in eight weeks to reassess response. If again there is no change or worsening, repeat ileocolonoscopy assessment and subsequent therapy discontinuation and switch should be considered. If there is IUS response (as defined above), then the interval to repeat IUS should be extended to 12 wk. Repeat IUS should be performed then every 12 wk until one year and if complete IUS transmural remission is achieved, then IUS can be performed every 6 mo for subsequent monitoring and confirmation of sustained transmural remission.

FUTURE APPLICATIONS

Eventually, the use of IUS at the point-of-care may be able to quantify the proportion of active *vs* chronic inflammation in patients with IBD. This will aid in treatment decision making, guiding choices toward either an anti-inflammatory or anti-fibrotic therapy, with or without surgery. In a study of 35 CD patients who underwent sheer wave elastography (SWE) within 1 wk of surgical resection, a cut-off value of 22.5 kPa was 69.6% sensitive and 91.7% specific with an AUROC of 0.82 to discriminate between mild/moderate and severe fibrosis based on histopathology[58]. Future studies validating cut-offs to discriminate degrees of fibrosis and active inflammation are still needed, but in the future provides will be able to personalize management for patients based on where they fall on the active inflammation *vs* fibrosis pathway utilizing SWE[59,60]. Additionally, use of hand-held IUS in combination with artificial intelligence will be able to help patients monitor both their active inflammation and fibrosis remotely for further tight control in the future[61].



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Figure 4 Intestinal ultrasound-based tight control monitoring algorithm. IUS: Intestinal ultrasound; BWT: Bowel wall thickness; CDS: Color Doppler signal.

CONCLUSION

IUS is an accurate, non-invasive cross-sectional imaging tool to assess IBD activity in real time. Incorporation of IUS into a tight control monitoring strategy promotes quick and impactful treatment decisions that can modify disease progression and enhance patient compliance. Specific populations of patients with IBD, especially children and pregnant women, would benefit significantly from the increased use of IUS for disease monitoring. Continued advances in technology will likely allow for enhanced stratification of active *vs* chronic inflammation at the point-of-care and enable remote monitoring for even tighter control. Ultimately, an IUS-based tight control monitoring algorithm and prediction tool may be used for early treatment decisions to achieve sustained deep remission and disease modification.

FOOTNOTES

Author contributions: Dolinger MT and Kayal M contributed to the conception of the manuscript, Dolinger MT drafted manuscript; Kayal M edited the manuscript.

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Mechanisms of gastrointestinal barrier dysfunction in COVID-19 patients

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Abstract

Coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a major global public health event, resulting in a significant social and economic burden. Although COVID-19 was initially characterized as an upper respiratory and pulmonary infection, recent evidence suggests that it is a complex disease including gastrointestinal symptoms, such as diarrhea, nausea, and vomiting. Moreover, it remains unclear whether the gastrointestinal symptoms are caused by direct infection of the gastrointestinal tract by SARS-CoV-2 or are the result of systemic immune activation and subsequent dysregulation of homeostatic mechanisms. This review provides a brief overview of the mechanisms by which SARS-CoV-2 disrupts the integrity of the gastrointestinal barrier including the mechanical barrier, chemical barrier, microbial barrier, and immune barrier.

Key Words: Gastrointestinal barrier dysfunction; SARS-CoV-2; COVID-19; Angiotensin-converting enzyme 2; Microbiome; Immune cells

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Core Tip: Coronavirus disease 2019 (COVID-19) has become a major global public health event, resulting in a significant social and economic burden. Although COVID-19 was initially characterized as an upper respiratory and pulmonary infection, recent evidence suggests that it is a complex disease including gastrointestinal symptoms. Moreover, it remains unclear whether the gastrointestinal symptoms are caused by direct infection of the gastrointestinal tract by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or are the result of systemic immune activation and subsequent dysregulation of homeostatic mechanisms. This review provides a brief overview of the mechanisms by which SARS-CoV-2 disrupts gastrointestinal barrier integrity.

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INTRODUCTION

Since the advent of coronavirus disease 2019 (COVID-19), the disease has spread globally and had a profound impact on the lives and health of people around the world[1]. The virus that causes COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), replicates and matures once it enters cells through the angiotensin-converting enzyme 2 (ACE2) receptor. SARS-CoV-2 can trigger an inflammatory response that involves the activation of immune cells by a variety of cytokines[2,3]. ACE2 receptors are present in multiple cell types throughout the body, including the oral and nasal mucosa, lung, and gastrointestinal tract, indicating that SARS-CoV-2 can cause multi-organ damage[4,5].

The intestinal tract is the digestive organ of the body, but also has endocrine and immune functions, and is the first line of defense against non-specific infections. However, the intestine is also the largest reservoir of bacteria and endotoxins in the body and is, therefore, a hidden source of infection. While digesting and absorbing nutrients, the intestine contains bacteria and their metabolites, and the gastrointestinal barrier plays a very important role in preventing systemic absorption of harmful microbes and substances[6,7]. The gastrointestinal barrier is composed of the intestinal epithelial cell layer, mucus layer, normal intestinal flora, intestinal immune system, and intestine-hepatic axis, which together perform the functions of mechanical barrier, chemical barrier, microbial barrier, and immune barrier (Figure 1). This barrier plays an important role in homeostasis by preventing harmful substances and pathogens in the gastrointestinal tract from entering the internal environment of the body[8]. In this review, we describe the pathophysiological mechanisms of gastrointestinal barrier dysfunction in COVID-19 patients (Figure 2).

MECHANISMS OF THE GASTROINTESTINAL BARRIER

Mechanical barrier

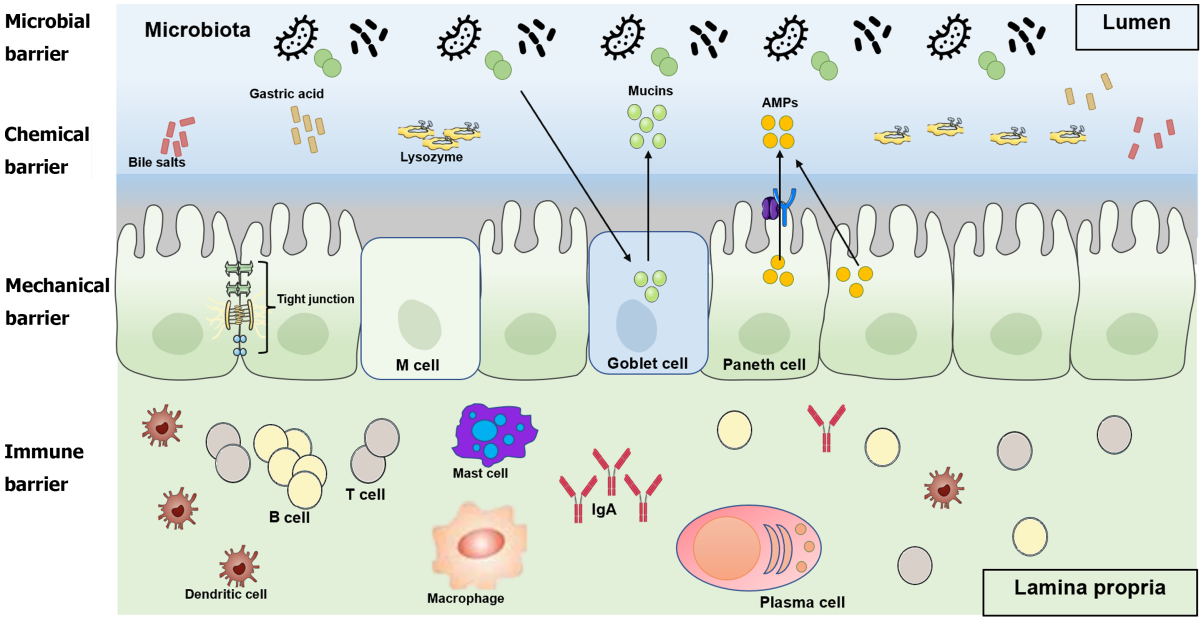
The intestinal mechanical barrier consists of intestinal mucosal epithelial cells, tight junctions (TJs) between epithelial cells, and a layer of bacteria and mucous on the surface of epithelial cells. TJs are the most important component of the intestinal mechanical barrier[9] and are composed of four transmembrane proteins: Zonula occludens (ZO); occludin; claudins, and junctional adhesion molecules (JAMs). TJs form an important barrier against the translocation of bacteria and toxins in the intestine [10]. The TJs between cells close the gaps between adjacent intestinal epithelial cells, preventing bacteria and antigens in the intestinal lumen from entering the lamina propria and activating immune cells in the lamina propria. Therefore, TJs maintain the stability of the intestinal mucosal barrier and prevent abnormal immune reactions in the mucosa. Moreover, the TJs between intestinal epithelial cells play an important role in maintaining the morphological structure of epithelial cells, regulating the differentiation and repair of epithelial cells and intercellular material transport, and maintaining the barrier function of the intestinal mucosa and intestinal mucosal permeability[10-12].

Chemical barrier

The intestinal chemical barrier, also called the mucus layer, is a general term for the antimicrobial substances produced by the resident intestinal bacteria and host cells *e.g.,* Paneth cells and the chemicals that inhibit bacterial adhesion and colonization, such as gastric acid, digestive enzymes, lysozyme, and mucin[13,14]. The mucus layer, which is mainly composed of mucins and secretory mucin MUC2, limits the contact of gastrointestinal tissues with the microbiota[15,16]. Therefore, the mucus layer separates bacteria from intestinal epithelial cells in the intestinal lumen while allowing nutrient absorption[17]. It also lubricates the luminal contents to prevent degradation of the gastrointestinal tissues[18,19]. Gastric acid acts mainly at the beginning of the small intestine to inactivate bacteria and other pathogenic microorganisms. Lysozyme exerts bactericidal and antibacterial effects on the intestinal epithelial surface and in the intestinal lumen[20,21]. Moreover, bile is an important chemical barrier to endotoxins, as bile salts bind to endotoxins in the intestine and prevent absorption from the intestine into the portal vein[22].

Microbial barrier

The normal intestine is inhabited by a large number of bacteria, with at least 500 different species, most of which are anaerobic[23]. Under normal conditions, the normal flora maintain a relatively stable proportional relationship with each other. They combine with the intestinal mucosa to form a micro-



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Figure 1 Schematic diagram of the intestinal barrier. The intestinal barrier is composed of biological, chemical, mechanical, and immune barriers. The intestinal lumen contains antimicrobial peptides, mucins, gastric acid, bile salts, lysozyme, and commensal bacteria, which together provide a protective barrier effect and inhibit pathogen colonization. The epithelial layer consists of a single layer of epithelial cells with tight junctions that prevent paracellular passage. In addition, this layer also harbors M cells, Goblet cells, and Paneth cells. The lamina propria contains a large number of immune cells, including B cells, T cells, plasma cells, macrophages, dendritic cells, and mast cells.

ecosystem that is both interdependent and interactive with the micro-spatial structure of the host and this micro-ecosystem forms the microbial barrier of the intestine[24]. Under normal microecological conditions, the dominant replication of non-pathogenic intestinal flora can hinder the survival of pathogenic bacteria. At the same time, non-pathogenic flora also secrete some antibacterial and antimicrobial substances, such as lactic acid and bacteriocins, which can interfere with and inhibit the vitality and function of pathogenic bacteria[25-27].

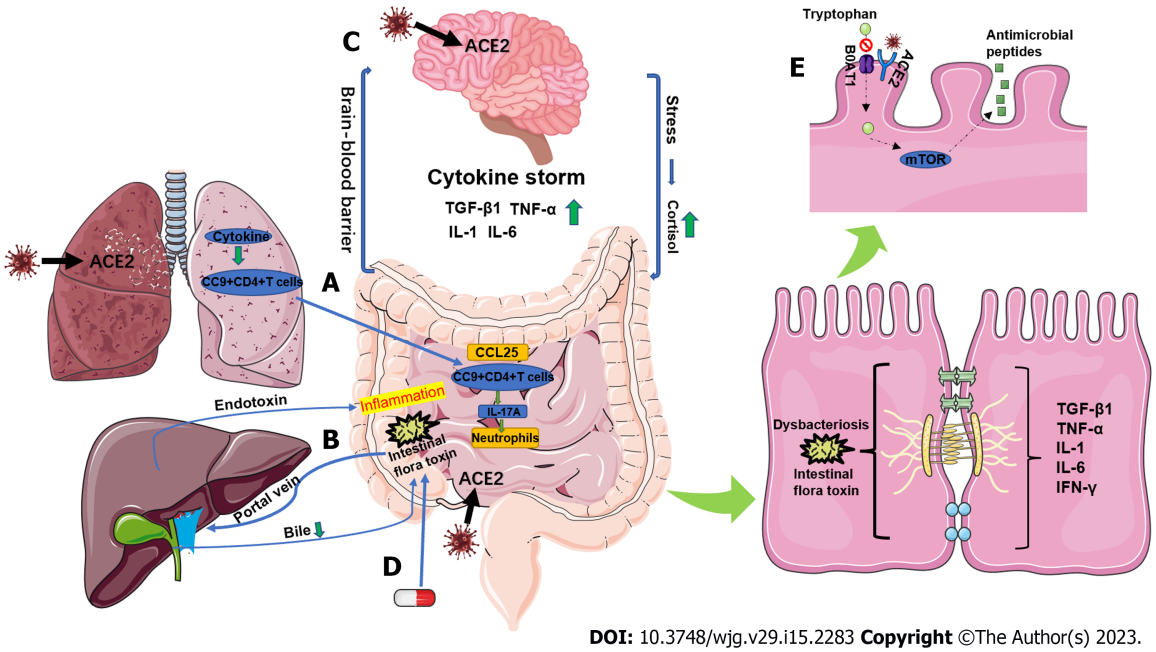
Immune barrier

The intestine is both a digestive organ and the largest immune organ in the body and is, therefore, an important part of the systemic immune system. The intestine is constantly exposed to antigenic substances, such as microbial antigens and food antigens, but the gastrointestinal barrier, including the immune barrier, can effectively prevent the penetration of these antigens. The intestinal immune defense system is mainly composed of gut-associated lymphoid tissue (GALT) in the intestinal wall and secreted IgA, IgM, and IgE. GALT is the site of induction and activation of the immune response, mainly in the Peyer's patches that are distributed throughout the small intestine[28,29]. Most of the IgA in intestinal secretions is secretory IgA (sIgA), which is the most secreted immunoglobulin in the body and the main immunoglobulin on the intestinal mucosal surface. sIgA plays a leading role in humoral immunity and is the first line of defense against the adhesion and colonization of pathogens in the intestinal mucosa[30,31]. Furthermore, perivascular macrophages in the lamina propria constitute an anatomical barrier that functions to prevent bacterial translocation[32]. Also, neutrophils play an important role in capturing and killing pathogens in enterocolitis[33].

FACTORS CONTRIBUTING TO THE GASTROINTESTINAL BARRIER DYSFUNCTION CAUSED BY SARS-COV-2

SARS-CoV-2 and the mechanical barrier

Damage to the gastrointestinal mechanical barrier can be caused by direct infection of intestinal cells with SARS-CoV-2. The spike protein of SARS-CoV-2 has a high affinity for ACE2, which is widely expressed in intestinal epithelial cells. SARS-CoV-2 enters target cells mainly through the ACE2 receptor thus causing primary damage to the intestine by altering the expression and function of TJ-related proteins, leading to disruption of the paracellular barrier function[34-37]. A study by Sun *et al*[38] that compared differentially enriched proteins in the stools of COVID-19 patients and normal participants found evidence of intestinal infection and intestinal damage caused by SARS-CoV-2. For example,



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Figure 2 Mechanisms of gastrointestinal barrier dysfunction in coronavirus disease 2019 patients. A: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds with angiotensin-converting enzyme 2 (ACE2) to enter the lung and, through the CCL25-CCR9 axis, mediate the recruitment of lung-derived effector CD4$^+$ T cells to the small intestine. This promotes the in-situ polarization of small intestinal Th17 cells and production of IL-17A leading to neutrophil aggregation and injuring the intestine; B: The intestinal flora is transferred to the liver through the portal vein, affecting liver function and leading to a decrease in endotoxin inactivation. Endotoxins enter the systemic circulation and induce an inflammatory response. In addition, decreased liver function also leads to reduced bile secretion and decreased inhibition of intestinal flora; C: The SARS-CoV-2 attack on cerebral neurons produces a large amount of pro-inflammatory cytokines that activate the systemic immune system, leading to damage of the gastrointestinal barrier. In addition, neurological damage activates the hypothalamic–pituitary–adrenal axis, which causes an increase in adrenal cortisol secretion, impairing the gastrointestinal barrier; D: The use of antibacterial or antiviral drugs may cause dysbiosis or immune suppression resulting in gastrointestinal barrier disorders; E: Competitive binding of ACE2 receptors by the SARS-CoV-2 virus inhibits tryptophan absorption through the B0AT1/ACE2 transport pathway in enterocytes, thus impairing regulation of antimicrobial peptide expression and causing dysbiosis of the flora, which disrupts the gastrointestinal barrier. ACE2: Angiotensin-converting enzyme 2.

certain protein components of human immunoglobulins and hemoglobin were upregulated in COVID-19 patients, suggesting an enhanced immune response and potential bleeding in the intestine.

Studies have also shown that ZO-1 interacts with: (1) Occludin, claudins, and JAM; (2) molecular components of intracellular TJ plaques, such as ZO-2, ZO-3, and cingulin; and (3) the actin cytoskeleton [39]. Thus, ZO-1 plays a key role in the structural and functional integrity of the paracellular barrier[39]. Furthermore, the cytoskeletal and barrier biomarkers KRT19 and ZO1 were respectively enriched in stool and blood samples from COVID-19 patients, suggesting increased intestinal paracellular permeability and injury[38,40]. Another group showed that the microbial metabolite d-lactate and the TJ regulator zonulin were increased in the serum of patients with severe COVID-19 and in COVID-19 patients with secondary infections[41]. These data suggest that biomarkers of intestinal permeability may also be early biomarkers for a fatal outcome. Indeed, treatment with larazotide, a zonulin inhibitor, significantly improved time-to-resolution of gastrointestinal symptoms and time-to-clearance of spike antigenemia in pediatric COVID-19 patients[42,43].

In addition, apoptosis of intestinal epithelial cells in COVID-19 patients is another important factor affecting the integrity of the gastrointestinal mechanical barrier. A study by Lehmann *et al*[44] demonstrated a significant increase in the number of apoptotic epithelial cells in COVID-19 patients by using immunohistochemistry to detect cleaved caspase-3. They also examined the histomorphological changes of epithelial cells and found apoptosis and subsequent regenerative proliferation of epithelial cells in the small intestine of COVID-19 patients[44].

In addition, infection of lung and small intestinal epithelial cells with SARS-CoV-2 stimulates secretion of large amounts of pro-inflammatory cytokines (chemokine-1, TGF-β1, TNF-α, IL-1, and IL-6) causing a cytokine storm and damage to the small intestine that disrupts the integrity of the gastrointestinal barrier[45]. Interestingly, this phenomenon was confirmed using human induced pluripotent stem (iPS)-derived intestinal epithelium, suggesting that iPSC could be a useful *in vitro* model for evaluating COVID-19 pathology in gastrointestinal barrier dysfunction[46].

SARS-CoV-2 and the chemical barrier

Many COVID-19 patients have a significantly reduced appetite, and severely affected patients, such as those dependent on mechanical ventilation, may not be able to eat, resulting in reduced secretion of gastric acid and bile. Under normal conditions, the intestinal epithelial surface is less susceptible to

damage by harmful substances due to the mucus cover, which plays an important role in the gastrointestinal barrier. Studies have shown that in rats receiving total parenteral nutrition, glands atrophy, the intestinal mucus layer is damaged and thinned, intestinal permeability increases, and the gastrointestinal barrier is disrupted[47,48]. In standard total parenteral nutrition, no food passes through the intestine in the loading state and certain essential intestinal nutrients such as glutamine are lacking. Numerous subsequent studies have confirmed that parenteral nutrition tends to damage the intestinal epithelial mucus layer, leading to disruption of the gastrointestinal barrier[49,50].

SARS-CoV-2 is known to be neuroinvasive, and when the body is infected with SARS-CoV-2, a cytokine storm is generated and the release of large amounts of pro-inflammatory cytokines leads to blood-brain barrier damage[51,52]. In addition, lipopolysaccharide (LPS) is recognized by Toll-like receptors, which transduce signals that stimulate release of inflammatory cytokines[53-55]. ACE2 is expressed in certain regions of the human brain as well as in neurons and SARS-CoV-2 can directly damage these target organs[56]. After SARS-CoV-2 infection, impairment of the gastrointestinal barrier causes leaky gut and increased translocation of bacterial metabolic components and toxins into the bloodstream, which activates the immune response and leads to systemic inflammation[57-59]. This can promote neuronal degeneration and the development of psychiatric and neurodegenerative disorders [60]. Furthermore, neurological damage can affect the gastrointestinal barrier *via* the neuroendocrine pathway; this occurs mainly through the hypothalamic–pituitary–adrenal (HPA) axis[61-63]. Stress activates the HPA axis, which ultimately leads to the release of glucocorticoids such as cortisol from the adrenal cortex. Cortisol can alter gastrointestinal dynamics, increase intestinal permeability, and affect the intestinal microbiota[61].

SARS-CoV-2 and the microbial barrier

When the microbial balance in the intestine is altered, pathogenic bacteria adhere to the intestinal epithelium and grow dominantly to replace the normal flora[64]. Pathogenic bacteria can directly damage the microvillous membrane proteins of the intestinal epithelium by producing bacterial proteases. Furthermore, pathogenic bacteria can alter biochemical reactions in the intestinal epithelium, leading to damage, blunting, fusion, or obliteration of the villi[65]. In addition, pathogenic bacteria can produce various toxins or other metabolites that inhibit protein synthesis in the intestinal epithelium, thereby damaging the intestinal mucosal barrier. Some opportunistic pathogens can also produce proteases that degrade IgA, which can weaken or eliminate the immune function provided by sIgA and promote bacterial translocation, leading to bacteremia and endotoxemia, thereby further damaging gastrointestinal barrier integrity[66].

A study by Sun *et al*[38] using metagenomic and metaproteomic methods showed significant changes in the composition of the gut microbiome in COVID-19 patients, characterized by a decrease in commensal species and an increase in opportunistic pathogenic species. Moreover, other studies have shown that the microbiota of COVID-19 patients is enriched with opportunistic pathogens, compared with healthy individuals[64,67-69]. Furthermore, the plasma concentration of the gut permeability marker FABP2 and gut microbial antigens PGN and LPS were significantly elevated in COVID-19 patients in comparison to healthy controls[70].

Hashimoto *et al*[71] found that ACE2 acts as a binding partner of amino acid transporter B0AT1 (SLC6A19) in the intestine and plays an important role in amino acid transport. Tryptophan is mainly absorbed through the B0AT1/ACE2 transport pathway in enterocytes, which activates the mammalian target of the rapamycin (mTOR) pathway to regulate the expression of antimicrobial peptides. These antimicrobial peptides affect the composition of the intestinal microbiota[71]. Competitive binding of ACE2 receptors by the SARS-CoV-2 virus prevents the absorption of tryptophan through the B0AT1/ACE2 transport pathway in enterocytes, impairs the regulation of antimicrobial peptide expression, and causes an intestinal dysbiosis, leading to disruption of the gastrointestinal barrier.

In addition, when patients with COVID-19 are admitted to hospital for treatment, changes in gastrointestinal barrier dysfunction are often induced by bacterial translocation and gut microbiome dysbiosis as a result of early and heavy use of antimicrobial drugs, such as macrolides, fluoroquinolones, or cephalosporin antibiotics[72-74]. Wu *et al*[75] reported that washed microbiota transplantation can improve the intestinal mucosal barrier function, inflammatory response, and immunity. Therefore, this treatment is expected to be an efficacious and safe therapeutic option for the treatment of COVID-19 patients with gut microbiota dysbiosis[75].

SARS-CoV-2 and the immune barrier

After binding to ACE2 receptors expressed on intestinal epithelial cells, SARS-CoV-2 is transported to the nuclear endosome and releases its RNA[76,77]. Toll-like receptors recognize viral RNA and signal downstream mediators, which induce IFN-α and -β production and then activate the transcription factor NF-κB to produce pro-inflammatory cytokines[78]. During SARS-CoV-2 infection, small intestinal tissue and feces show increased pro-inflammatory markers, including neutrophil and monocyte accumulation, increased chemokine-1, TGF-β1, IL-1, IL-6, IL-8, and IFN-γ expression and decreased levels of the anti-inflammatory cytokine IL-10[45,79,80]. IFN-γ is produced by several types of immune cells, especially T helper type 1 (Th1) cells. IFN-γ acts as a major inducer of the cell-mediated response to infection by activating macrophages, enhancing antigen presentation and T cell differentiation[81], and interacting

directly with epithelial cells, leading to chemokine expression and antimicrobial peptide secretion[82, 83]. In addition, mucosal $CD4^+$ and $CD8^+$ T cells, Th17 cells, neutrophils, dendritic cells, and macrophages were activated and T regulatory cells were reduced after intestinal epithelial cells were infected by SARS-CoV-2. Therefore, SARS-CoV-2 infection promotes an over-activated immune response that further damages intestinal epithelium[84-86]. Interestingly, one study found that SARS-CoV-2 was not always measurable in the stools of COVID-19 patients with gastrointestinal symptoms. These data further suggest that the gastrointestinal barrier dysfunction may not be a direct result of viral infection of intestinal mucosal epithelial cells, but rather a result of the immune response[87].

CCL25 is expressed in enterocytes and the CCL25-CCR9 axis mediates recruitment of lung-derived effector $CD4^+$ T cells to the small intestine[88]. $CCR9^+CD4^+$ T cells disrupt homeostasis of the intestinal flora, thereby promoting the in-situ polarization of small intestinal Th17 cells and the production of IL-17A, leading to neutrophil aggregation and ultimately mediating intestinal immune-mediated injury[88, 89]. Moreover, damage to the intestinal mucosa and bacterial imbalance can lead to harmful metabolites in the intestine being transferred to the liver through the portal vein, affecting liver function[90]. With impaired liver function, endotoxin inactivation is reduced, resulting in endotoxin entering the systemic circulation[91-94]. This induces an inflammatory response, damages intestinal mucosa, and causes bacterial translocation and bacteremia, inducing a systemic inflammatory response and thus forming a vicious cycle[95].

Furthermore, antiviral drugs can also cause gastrointestinal barrier dysfunction. For example, severe and persistent diarrhea may be associated with the use of oseltamivir and arborol. Other drugs that can cause gastrointestinal barrier dysfunction include chloroquine phosphate, lopinavir, remdesivir, and some proprietary Chinese medicines, as well as immunosuppressors[96].

CONCLUSION

Gastrointestinal barrier dysfunction in COVID-19 patients is not an independent symptom, but rather a trigger of other diseases. In critically ill patients, gastrointestinal barrier dysfunction, if not treated early, may aggravate primary disease or even cause multi-organ dysfunction syndrome, thus endangering patients' lives. To assess gastrointestinal barrier dysfunction in COVID-19 patients, clinicians should not only consider the effect of the primary disease, but also the pathophysiological situation within the intestine. The pathological mechanisms that cause gastrointestinal barrier damage and dysfunction are complex and various mechanisms are often intertwined, interacting with each other. Therefore, the clinical diagnosis and treatment of gastrointestinal barrier dysfunction should be comprehensive and targeted.

FOOTNOTES

Author contributions: Xue W and Honda M designed the research; Xue W and Honda M performed the research; Xue W, Honda M and Hibi T analyzed the data; Xue W and Honda M wrote the paper.

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ORIGINAL ARTICLE

Basic Study

Ferroptosis inhibition attenuates inflammatory response in mice with acute hypertriglyceridemic pancreatitis

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Abstract

BACKGROUND

Ferroptosis is involved in developing inflammatory diseases; yet, its role in acute hypertriglyceridemic pancreatitis (HTGP) remains unclear.

AIM

To explore whether ferroptosis is involved in the process of HTGP and elucidate its potential mechanisms.

METHODS

An HTGP mouse model was induced using intraperitoneal injection of P-407 and caerulein (CAE). Then, pancreatic tissues from the model animals were subjected to proteome sequencing analysis. The pathological changes and scores of the pancreas, lung, and kidney were determined using hematoxylin-eosin staining. The levels of serum amylase (AMY), triglyceride, and total cholesterol were measured with an automatic blood cell analyzer. Additionally, the serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were determined by enzyme linked immunosorbent assay. Malonaldehyde (MDA), glutathione (GSH), and Fe^{2+} were detected in the pancreas. Finally, immunohistochemistry was performed to assess the expression of ferroptosis-related proteins.

RESULTS

Proteome sequencing revealed that ferroptosis was involved in the process of HTGP and that NADPH oxidase (NOX) 2 may participate in ferroptosis

regulation. Moreover, the levels of serum AMY, TNF-α, IL-6, and IL-1β were significantly increased, MDA and Fe^{2+} were upregulated, GSH and ferroptosis-related proteins were reduced, and the injury of the pancreas, lung, and kidney were aggravated in the P407 + CAE group compared to CAE and wild type groups (all $P < 0.05$). Notably, the inhibition of ferroptosis and NOX2 attenuated the pathological damage and the release of TNF-α, IL-6, and IL-1β in the serum of the mice.

CONCLUSION

Ferroptosis was found to have an important role in HTGP and may be considered a potential target for clinical treatment.

Key Words: Ferroptosis; Acute hypertriglyceridemic pancreatitis; NADPH oxidase 2; Ferrostatin-1; Vas2870

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Core Tip: Ferroptosis is involved in the development of inflammatory diseases, but its relationship with acute hypertriglyceridemic pancreatitis (HTGP) remains unclear. This study discoverd ferroptosis is involved in the process of HTGP and therefore a potential target for clinical treatment.

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INTRODUCTION

Hypertriglyceridemia is one of the leading causes of acute pancreatitis (AP). In China, the incidence rate of acute hypertriglyceridemic pancreatitis (HTGP) has increased from 13% in 2009 to 25.6% in 2013, and it is currently considered a second predominant type of AP[1-3]. Compared with AP caused by other causes, HTGP is associated with multiple organ dysfunction syndrome (MODS) and higher mortality[1, 4]. However, the underlying mechanisms of HTGP remain clear, and no effective treatments are available. Therefore, exploring the pathogenesis of HTGP and seeking more accurate therapeutic targets are of critical importance.

It is generally believed that excessive triglyceride (TG), which is hydrolyzed by pancreatic lipase, results in the release of damage-inducing free fatty acids (FFA), which then directly damage the pancreatic function and induce and amplify inflammatory responses through oxidative stress, aggravating multiple organ damage and leading to MODS[5-8]. In 2020, Rawla *et al*[9] found that when serum TG levels were higher than 1000 mg/dL, the incidence of AP was 5%, but when TG was over 2000 mg/dL, the incidence of AP increased to 10%-20%[9].

Previous studies have also revealed that various forms of regulated cell death (RCD), such as apoptosis and autophagy, are involved in the pathogenesis of AP[10-12]. Yet, the role of ferroptosis in HGAP is still not fully understood. Ferroptosis, as an iron-dependent form of non-apoptotic cell death first reported in 2012, is induced by excessive accumulation of peroxidized lipids and strictly regulated by glutathione peroxidase 4 (GPX4) and acyl-CoA synthetase long-chain family member 4[13-16]. Existing evidence suggests that the development of many diseases, including cancer, neurological diseases, ischemia-reperfusion injury, kidney injury, and cardiovascular diseases, is driven by ferroptosis[17-21]. In addition to altered iron homeostasis, the excessive production of reactive oxygen species (ROS) has also been reported as a key factor in ferroptosis induction[22].

Abnormal lipid metabolism is also considered to have an important role in the process of HTGP[4]. Meanwhile, FFA has been found to induce the excessive release of ROS and accumulation in the body [5]. Therefore, since abnormal lipid metabolism and ROS accumulation occur in both ferroptosis and HTGP, we hypothesized that ferroptosis might also participate in the process of HTGP. In the present study, we assessed the role of ferroptosis in HTGP through proteome sequencing. Then, we investigated the possible pathways and verified our results using animal experiments. This study provides new insides into the mechanism and treatment of HTGP.

MATERIALS AND METHODS

Materials and reagents

P-407 and caerulein (CAE) were purchased from Sigma Chemical (St. Louis, MO, United States). Ferrostatin-1 (Fer-1) and Vas2870 (Vas) were obtained from MedChemExpress (MCE, Monmouth Junction, NJ, United States). P-407 was solubilized in phosphate buffer solution (PBS) and stored at 4 °C. CAE, Fer-1, and Vas were solubilized in dimethyl sulfoxide and stored at 20 °C. Enzyme linked immunosorbent assay (ELISA) kits were obtained from Solarbio (Beijing, China).

Animals and modeling

Adult male C57BL/6 mice (25-30 g, 8-wk) were housed in a ventilated environment with a temperature of 22 ± 1 °C, relative humidity of 50% ± 1%, and a light/dark cycle of 12/12 hr and given water and food ad libitum. After acclimatization, mice were randomly categorized into 4 groups (n = 4 per group): Control, P-407, AP (CAE), and HTGP (P-407 + CAE). The P-407 exposure group was intraperitoneally injected with a dosage of 0.6 g/kg body weight (bw) P-407[1,23]. Eight hours later, the AP group was intraperitoneally injected with a dosage of 100 μg/kg bw CAE 8 times (once every hour)[1,24]. The control group was injected with the same volume of PBS. After the last injection, all mice were alive. The animals were sacrificed and pancreas, lung, and kidney tissues were excised.

Serum assays

After the mice fasted for 16 h, Roche blood glucose meter was used to detect fasting blood glucose, and the collected blood from eyeballs was centrifuged at 4 °C, 3000 g for 10 min. The activities of the serum α-amylase (AMY), total cholesterol (TC), TG, aspartate transaminase (AST), and alanine aminotransferase (ALT) in blood samples were measured by an automatic blood cell analyzer (Mindray, Shenzhen, China). Malonaldehyde (MDA), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were detected by specific ELISA kits (Solarbio Science & Technology Co., Ltd, Beijing, China), following the manufacturer's instructions. The concentrations were expressed as pg/mL. The serum fasting insulin (FINS) levels were detected by a specific ELISA kit (Proteintech, Wuhan, China). The homeostasis model assessment insulin resistance index (HOMA-IR) = fasting blood glucose (FBG, mmol/L) × Fasting insulin (FINS, mIU/L)/22.5.

Glutathione detection

The Micro Reduced glutathione (GSH) Assay Kit was from Solarbio (Solarbio Science & Technology Co., Ltd, Beijing, China). Continuously dilute the standard product according to the corresponding concentration, and make the standard curve. Then, the supernatant of pancreas samples and total glutathione test solution were added into the 96-well plate successively, mixed, and incubated at 25 °C or room temperature for 5 min. 50 μL 0.5 mg/mL NADPH solution was added to per hole and mixed. The absorbance of each hole was measured immediately with an enzyme marker (λ = 412 nm). The total GSH content of each pore was obtained according to absorbance and standard curve. The sample is processed with GSH scavenger and GSH scavenger assistant in the kit to remove GSH, and the content of oxidized glutathione disulfide (GSSG) is measured, finally the content of reduced GSH is subtracted by total GSH and GSSG.

Fe^{2+} detection

Fe^{2+} in pancreas was detected according to the instructions in the Ferrous Ion Content Assay Kit (Solarbio Science & Technology Co., Ltd, Beijing, China). The absorbance was measured immediately with an enzyme marker (λ = 593 nm). The content of Fe^{2+} was obtained according to the concentration of standard substance and absorbance.

Malondialdehyde detection

MDA in pancreas was detected according to the instructions of MDA Content Assay Kit (Solarbio Science & Technology Co., Ltd, Beijing, China). After pretreatment, appropriate amount of supernatant was taken, and the absorbance of each group was measured at 532 nm. MDA content was obtained according to absorbance.

Histology and immunohistochemistry analyses

The tissue sections (pancreas, lung, and kidney) were cut into continuous slices. Hematoxylin–eosin (HE) staining was performed according to the Solarbio kit guidelines, and immunohistochemistry (IHC) analysis was conducted according to the Kangwei kit instructions. Images were acquired by Pannoramic MIDI (3DHISTECH). Brown-stained areas indicated immunoreactivity. Tissue sections were blindly evaluated by two pathologists based on previously reported criteria: The pathological injury of the pancreas was scored according to the edema, inflammation, hemorrhage, and necrosis of the pancreas [25]; the pathological score of the lung was measured on the basis of edema, neutrophil infiltration, and hemorrhage[26]; the pathological renal score was evaluated by the degree of tubulointerstitial injury

based on previously reported criteria[27].

Ferroptosis-related proteins (the light chain subunit solute carrier family 7 member 11 (SLC7A11) and GPX4 were determined by IHC analysis. Image J was used to quantify the expression of SLC7A11 and GPX4.

Protein extraction

The sample was ground with liquid nitrogen into cell powder and then transferred to a 5-mL centrifuge tube. After that, four volumes of lysis buffer (8 M urea, 1% protease inhibitor cocktail) were added to the cell powder, followed by sonication three times on ice using a high-intensity ultrasonic processor (Scientz). Next, the remaining debris was removed by centrifugation at 12000 g at 4 °C for 10 min. Finally, the supernatant was collected, and the protein concentration was determined with a Bicinchoninic Acid Assay kit according to the manufacturer's instructions.

Trypsin digestion

For digestion, the protein solution was reduced by 5 mmol/L dithiothreitol for 30 min at 56 °C and alkylated with 11 mmol/L iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mmol/L tetraethyl ammonium bromide to urea concentration < 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h digestion. Finally, the peptides were desalted using the C18 SPE column.

4D mass spectrometer

The tryptic peptides were dissolved in solvent A (0.1% formic acid, 2% acetonitrile/in water) and directly loaded onto a homemade reversed-phase analytical column (25 cm length, 75/100 μm inside diameter). Peptides were separated with a gradient from 6% to 24% solvent B (0.1% formic acid in acetonitrile) over 70 min, 24% to 35% in 14 min, and climbing to 80% in 3 min, then holding at 80% for the last 3 min, all at a constant flow rate of 450 nL/min on a nanoElute UHPLC system (Bruker Daltonics).

The peptides were subjected to a capillary source followed by the timsTOF Pro (Bruker Daltonics) mass spectrometry. The electrospray voltage applied was 1.60 kV. Precursors and fragments were analyzed at the TOF detector, with an MS/MS scan ranging from 100 to 1700 m/z. The timsTOF Pro was operated in parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 0 to 5 were selected for fragmentation, and 10 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 30 s.

Database search

The resulting MS/MS data were processed using the MaxQuant search engine (v.1.6.15.0). Tandem mass spectra were searched against the mouse SwissProt database (17089 entries) concatenated with the reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and acetylation on protein N-terminal and oxidation on Met were specified as variable modifications. The false discovery rate was adjusted to < 1%. The data base is UniProt, the species-specific protein library is Mus_musculus_10090_SP_2021072. fasta. The proteomic analysis was performed by PTM Biolabs.

Principle component analysis

Principle component analysis (PCA) was conducted using R packages 'gmodels', 'ggplot2', and 'ggrepel'. The protein quantitative principal component (PC) analysis results of all samples were calculated. The degree of aggregation between samples in the figure represents the difference between samples.

Differentially expressed proteins analysis

The samples to be compared were selected, and the relative quantitative values of each protein in the repeated samples were taken as the difference multiple (fold change, FC). In order to judge the significance of the difference, the relative quantitative value of each protein in the samples of the comparison group was subjected to a *t*-test. The corresponding *P* value was calculated as the significance index. The default *P* value was < 0.05.

Gene Ontology enrichment analysis

Gene Ontology (GO) is used to express various properties of genes and gene products. GO annotations are divided into three categories: Biological Process, Cellular Component, and Molecular Function, which aim to explain the biological role of proteins from different perspectives. We performed differential protein enrichment analysis on a biological process in GO classification.

Kyoto Encyclopedia of Genes and Genomes pathway analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG)[28] analysis was conducted using the 'clusterProfiler' package[29] of R software. A previously described method was used[30].

Cluster analysis

According to Fisher's exact test obtained by the enrichment analysis, the related functions in different comparison groups were clustered using the hierarchical clustering method, and heatmaps were drawn. In the heatmaps, the horizontal direction of the heatmap represents different comparison groups, and the vertical direction indicates related functions enriched by differentially expressed proteins in different comparison groups. The color patches corresponding to the functional description of the enrichment of the differentially expressed proteins in different comparison groups indicate the degree of enrichment. Red color represents strong enrichment, and blue represents weak enrichment.

Statistical analysis

All data are expressed as mean ± SD. The statistical analyses were conducted using SPSS version 18.0 software, and the differences in data among the groups were analyzed using the unpaired Student *t*-test of variance test. $P < 0.05$ (2-tailed) was considered to indicate a statistically significant difference.

RESULTS

P-407 and CAE were successfully used to develop the mouse model of HTGP

The exposure of mice to P-407 and CAE for 48 h resulted in much higher TC and TG in the P-407 (model for hyperlipidemia) and P-407 + CAE (model for HTGP) groups than in the Ctrl and CAE (model for AP) groups (Figure 1A). A slight rise in the AMY level was seen in the CAE group, whereas in the P-407 + CAE group, a significant increase in the AMY level was detected compared to other groups (all $P < 0.05$, Figure 1B). In addition, an increase in AST and ALT levels were seen in the CAE-treated groups, but there was no difference between CAE and P-407 + CAE-treated groups ($P > 0.05$, Figure 1C). There was no change in HOMA-IR among different groups ($P > 0.05$, Figure 1D). Furthermore, in the CAE group, a minor injury was seen in the pancreas and kidney, whereas in the P-407 + CAE group, serious injuries were observed in the pancreas, lung, and kidney, which were detected by HE staining (Figure 1E-G). Also, in the P-407 + CAE group, high levels of serum TNF-α, IL-1β, and IL-6 were detected (Figure 1H). These results proved that P-407 and CAE mice models were successful.

Proteome profiling predicts the role of ferroptosis in P-407 + CAE-induced HTGP rather than AP

Next, proteome sequencing was used to compare the mechanisms of HTGP and AP. Samples of the same group were clustered together from both PCA and clustering analyses (Figure 2A and B). The CAE treatment caused the rightward movement of samples along the PC1, explaining 56.0% of the total variance. The P-407 + CAE treatment upward along PC2 explained 17.5% of the total variance (Figure 2A). Additionally, 190 proteins were downregulated, whereas 272 proteins were upregulated in the pairwise comparisons (CAE *vs* WT). Furthermore, 43 proteins were downregulated and 125 were upregulated when comparing P-407 + CAE and CAE groups (Figure 2C and D). These results suggested that AP and HTGP exert different effects on the pancreas.

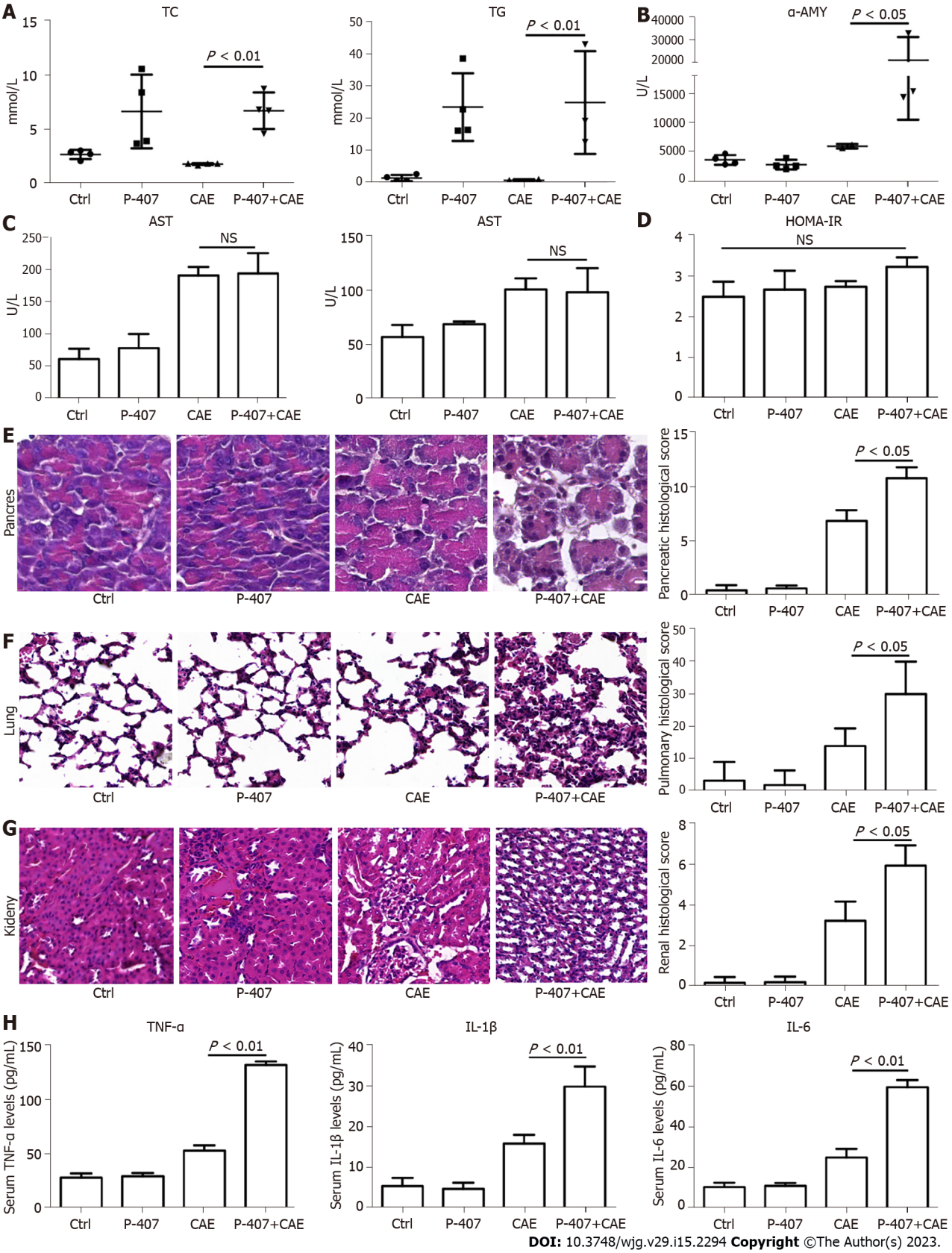
GO analyses further showed that the acute inflammatory response was highly enchased in the P-407 + CAE group *vs* CAE group; this difference, however, was not evident when comparing CAE *vs* WT groups, which also confirmed that P-407 + CAE induces more severe pancreas injury than CAE (Figure 2E). Furthermore, KEGG pathway analyses also showed that upregulated proteins were weakly enriched for ferroptosis in the CAE group *vs* WT group but also strongly enriched for ferroptosis in the P-407 + CAE group *vs* CAE group (Figure 2F). These results suggest that ferroptosis occurs specifically in P-407 + CAE-induced HTGP.

Verification of P-407 + CAE-induced ferroptosis in mice

To verify the results of proteome sequencing, we performed IHC staining of mouse pancreatic tissue. The SLC7A11 and GPX4 expression levels were significantly lower in the P-407 + CAE group than in the CAE group (all $P < 0.05$, Figure 3A and B). The MDA (Figure 3C) levels in the P-407 + CAE group were significantly higher than those in the CAE group ($P < 0.05$), while the GSH level in the P-407 + CAE group was significantly lower than that in CAE group ($P < 0.05$) (Figure 3D). The Fe^{2+} (Figure 3E) levels in the P-407 + CAE group were significantly higher than those in the CAE group ($P < 0.05$), too. This data indicates that P-407 + CAE induces ferroptosis in the pancreas of the mice.

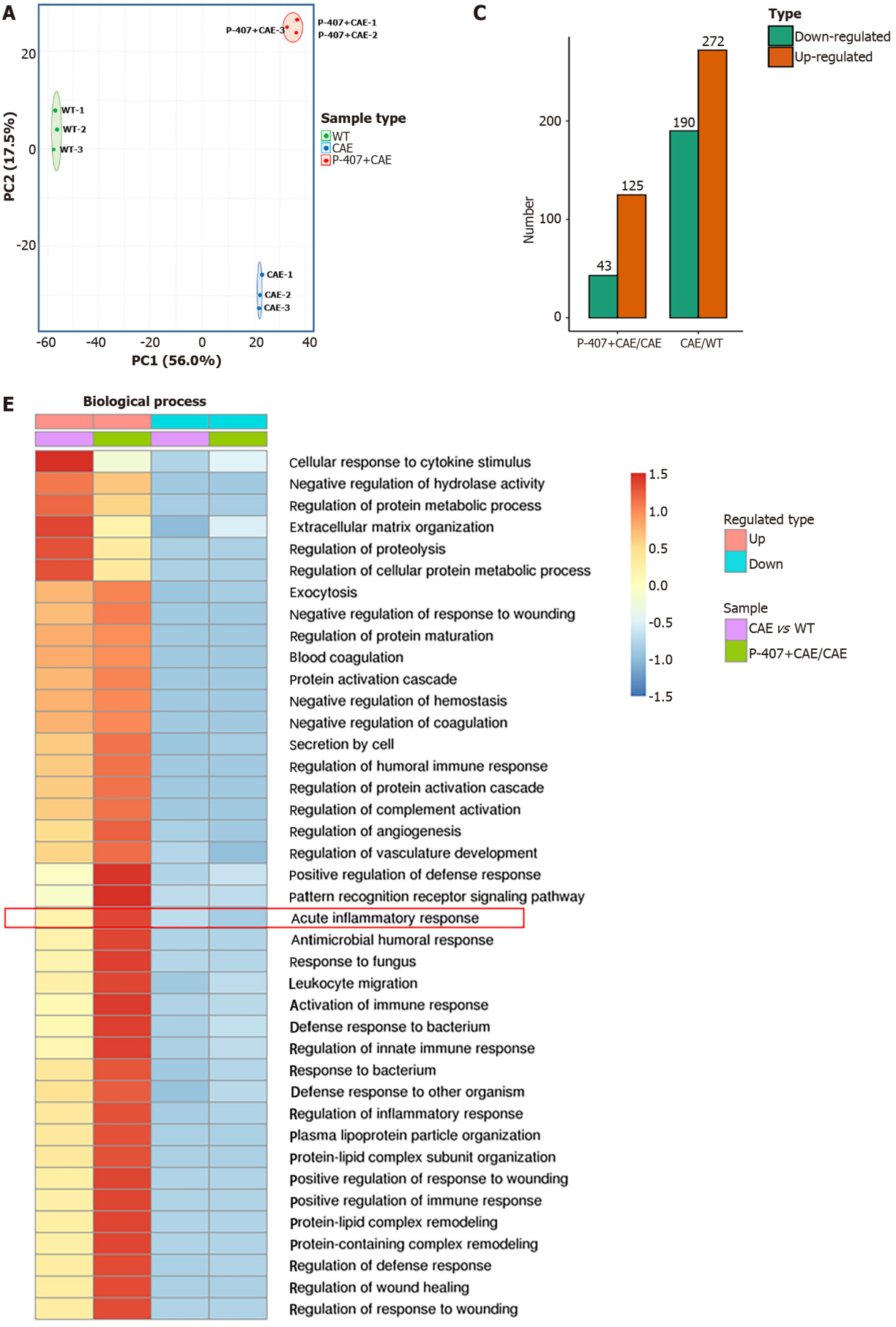
HTGP can be attenuated through ferroptosis inhibition in mice

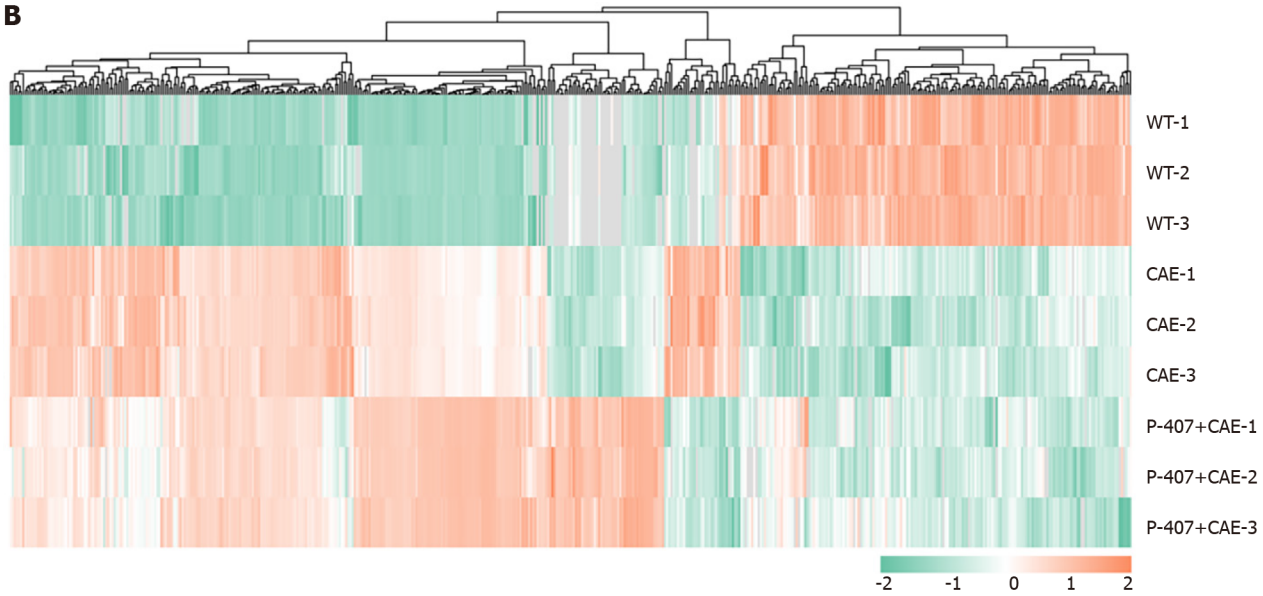
Since we had established that P-407 + CAE specifically induced ferroptosis in the pancreas of the mice, we further aimed to determine the role of ferroptosis in the regulation of HTGP. First, we used Fer-1 to inhibit ferroptosis[7,8]. The expression of GPX4 (Figure 4A) in the P-407 + CAE + Fer-1 group increased

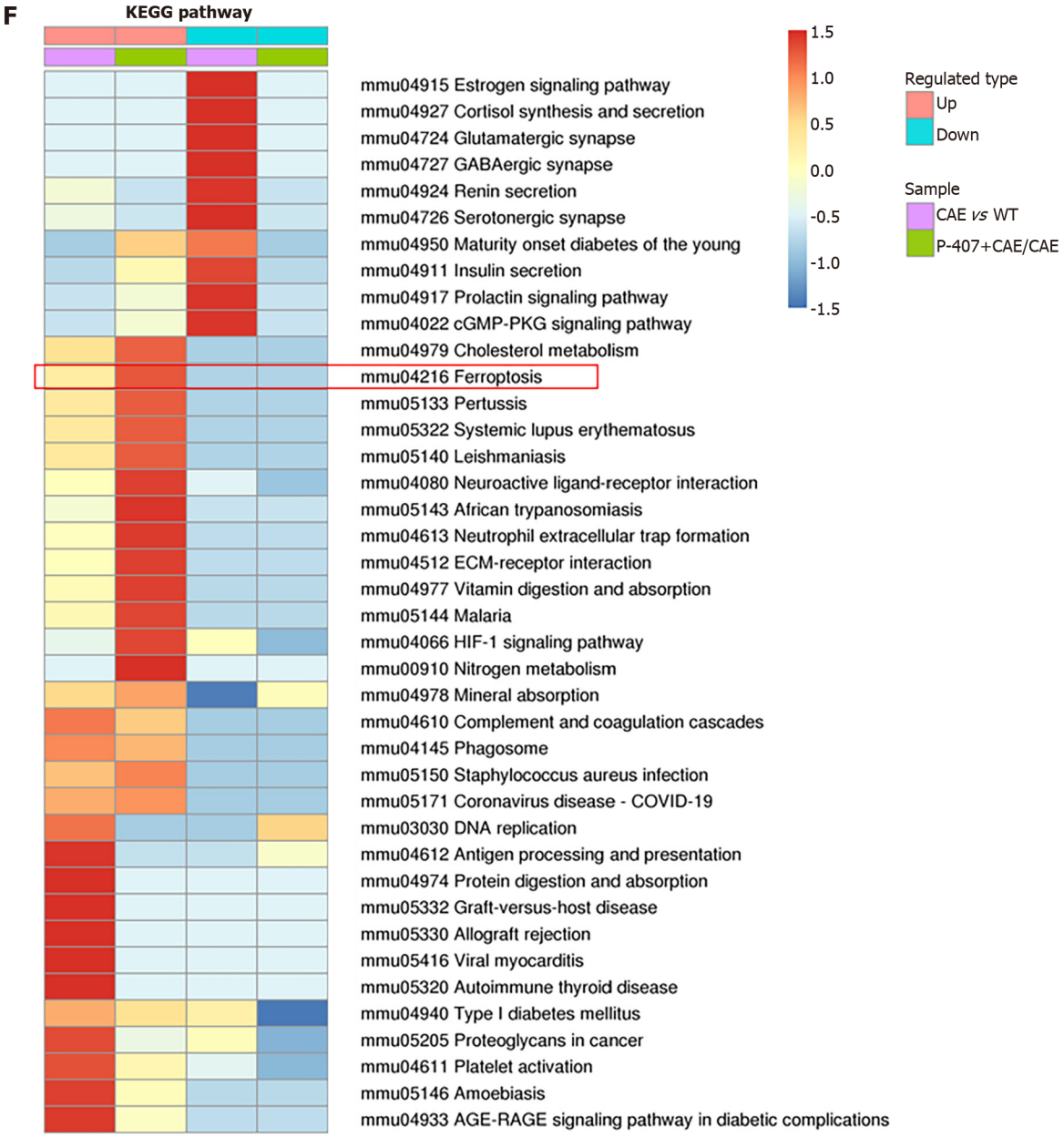


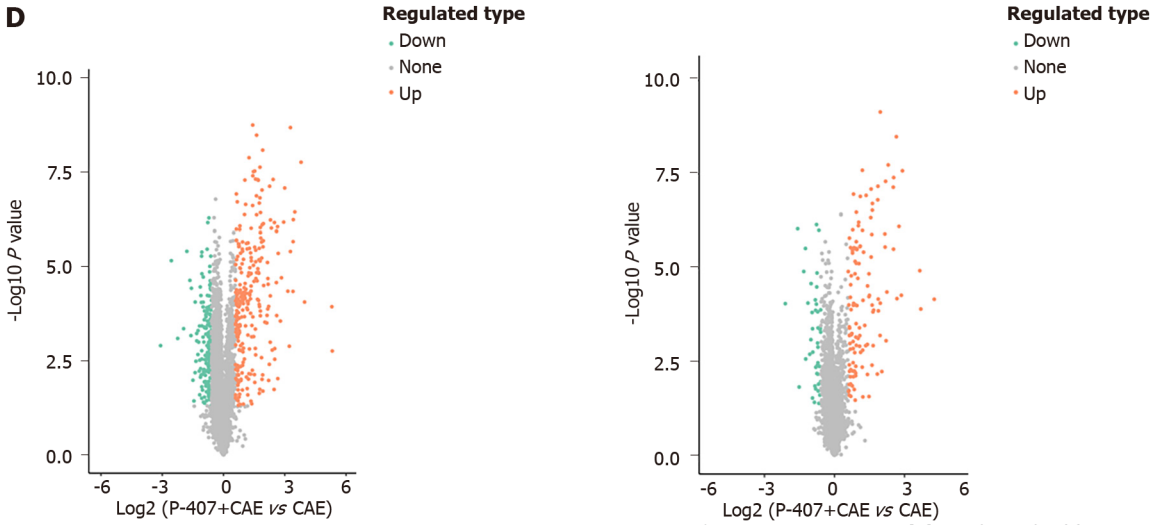
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Figure 1 P-407 and caerulein-induced hypertriglyceridemic pancreatitis in mice (*n* = 4). A: Serum total cholesterol and triglyceride levels; B: Serum amylase; C: Alanine aminotransferase and Alanine aminotransferase levels; D: Homeostasis model assessment insulin resistance index levels in mice exposed to P-407 and caerulein (CAE); E: Hematoxylin–eosin (HE) staining and histological scores of the pancreas; F: HE staining and histological scores of the lung; G: HE staining and histological scores of the kidney; H: Serum Tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 levels after P-407 and CAE exposure. AST: Aspartate transaminase; ALT: Alanine aminotransferase; HOMA-IR: Homeostasis model assessment insulin resistance index; HE: Hematoxylin–eosin; CAE: Caerulein; AMY: Amylase; TNF: Tumor necrosis factor; IL: Interleukin; NS: Not significant.


A
P-407+CAE-1
P-407+CAE-2
P-407+CAE-3
WT-1
WT-2
WT-3
CAE-1
CAE-2
CAE-3
Sample type
WT
CAE
P-407+CAE
PC2 (17.5%)
PC1 (56.0%)
C
Type
Down-regulated
Up-regulated
Number
43
125
190
272
P-407+CAE/CAE
CAE/WT
E
Biological process
Cellular response to cytokine stimulus
Negative regulation of hydrolase activity
Regulation of protein metabolic process
Extracellular matrix organization
Regulation of proteolysis
Regulation of cellular protein metabolic process
Exocytosis
Negative regulation of response to wounding
Regulation of protein maturation
Blood coagulation
Protein activation cascade
Negative regulation of hemostasis
Negative regulation of coagulation
Secretion by cell
Regulation of humoral immune response
Regulation of protein activation cascade
Regulation of complement activation
Regulation of angiogenesis
Regulation of vasculature development
Positive regulation of defense response
Pattern recognition receptor signaling pathway
Acute inflammatory response
Antimicrobial humoral response
Response to fungus
Leukocyte migration
Activation of immune response
Defense response to bacterium
Regulation of innate immune response
Response to bacterium
Defense response to other organism
Regulation of inflammatory response
Plasma lipoprotein particle organization
Protein-lipid complex subunit organization
Positive regulation of response to wounding
Positive regulation of immune response
Protein-lipid complex remodeling
Protein-containing complex remodeling
Regulation of defense response
Regulation of wound healing
Regulation of response to wounding
Regulated type
Up
Down
Sample
CAE *vs* WT
P-407+CAE/CAE

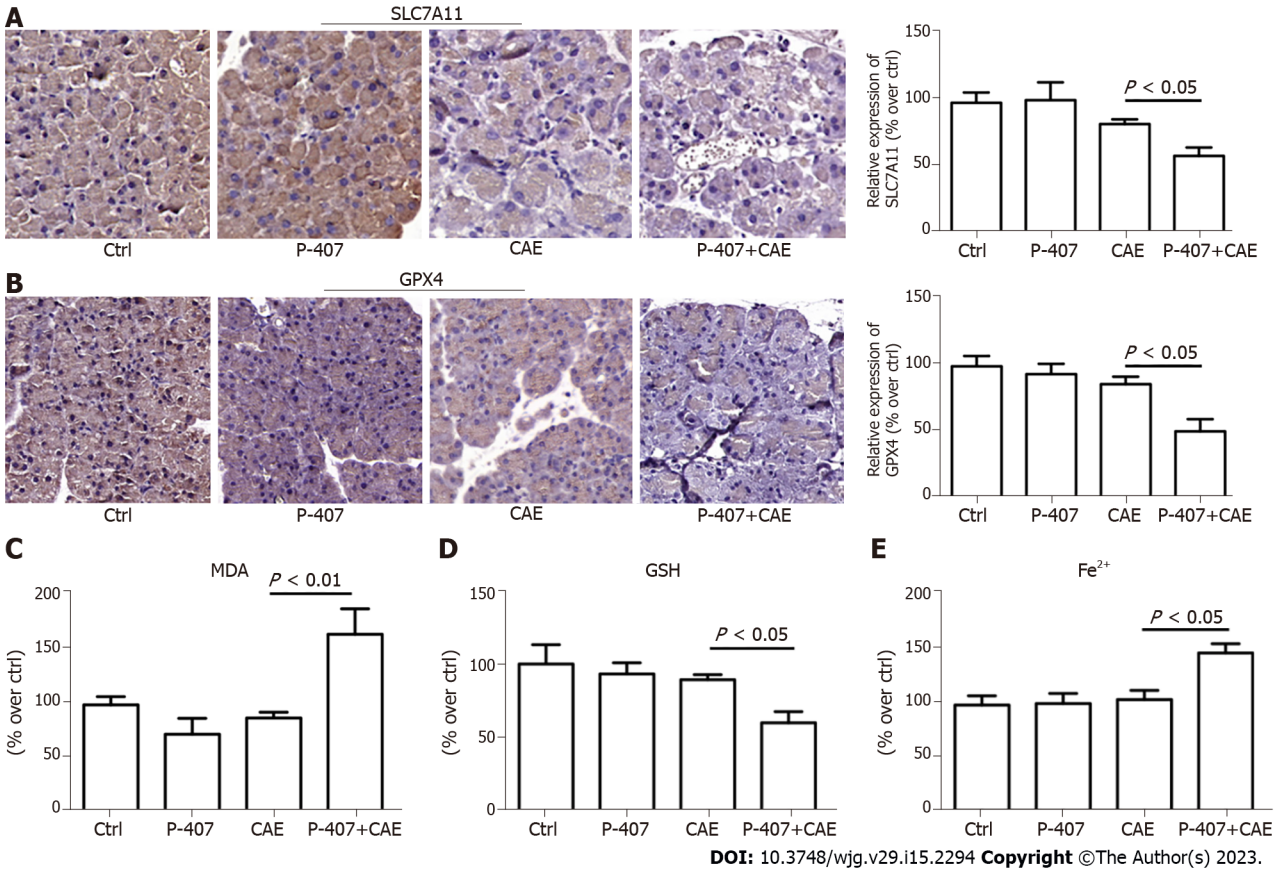

B
WT-1
WT-2
WT-3
CAE-1
CAE-2
CAE-3
P-407+CAE-1
P-407+CAE-2
P-407+CAE-3
-2
-1
0
1
2


F
KEGG pathway
mmu04915 Estrogen signaling pathway
mmu04927 Cortisol synthesis and secretion
mmu04724 Glutamatergic synapse
mmu04727 GABAergic synapse
mmu04924 Renin secretion
mmu04726 Serotonergic synapse
mmu04950 Maturity onset diabetes of the young
mmu04911 Insulin secretion
mmu04917 Prolactin signaling pathway
mmu04022 cGMP-PKG signaling pathway
mmu04979 Cholesterol metabolism
mmu04216 Ferroptosis
mmu05133 Pertussis
mmu05322 Systemic lupus erythematosus
mmu05140 Leishmaniasis
mmu04080 Neuroactive ligand-receptor interaction
mmu05143 African trypanosomiasis
mmu04613 Neutrophil extracellular trap formation
mmu04512 ECM-receptor interaction
mmu04977 Vitamin digestion and absorption
mmu05144 Malaria
mmu04066 HIF-1 signaling pathway
mmu00910 Nitrogen metabolism
mmu04978 Mineral absorption
mmu04610 Complement and coagulation cascades
mmu04145 Phagosome
mmu05150 Staphylococcus aureus infection
mmu05171 Coronavirus disease - COVID-19
mmu03030 DNA replication
mmu04612 Antigen processing and presentation
mmu04974 Protein digestion and absorption
mmu05332 Graft-versus-host disease
mmu05330 Allograft rejection
mmu05416 Viral myocarditis
mmu05320 Autoimmune thyroid disease
mmu04940 Type I diabetes mellitus
mmu05205 Proteoglycans in cancer
mmu04611 Platelet activation
mmu05146 Amoebiasis
mmu04933 AGE-RAGE signaling pathway in diabetic complications
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Regulated type
Up
Down
Sample
CAE *vs* WT
P-407+CAE/CAE



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Figure 2 Effects of P-407 and caerulein on the pancreas revealed by proteome sequencing (*n* = 3). A: Principal component analysis; B: Heatmap showing relationships among different sample groups; C: Exhibition of changed protein numbers among the sample groups; D: Volcano plots showing proteins differentially expressed between sample groups; E: Biological process; F: Kyoto Encyclopedia of Genes and Genomes pathways enriched with proteins differentially expressed in pair-wise comparisons of 'CAE group' *vs* WT group'' and 'P-407 + CAE group' *vs* CAE group'. CAE: Caerulein; WT: Wide type; PC: Principal component.



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Figure 3 P-407 + Caerulein-induced pancreas ferroptosis in mice as established by immunohistochemistry (*n* = 4). A: Solute carrier family 7 member 11 in the pancreas tissues of the P-407 + Caerulein (CAE) group; B: Glutathione peroxidase 4 in the pancreas tissues of the P-407 + CAE group. The scale bar represents 50 mm; C: Malonaldehyde levels; D: Glutathione levels; E: Fe^{2+} levels in the pancreases of the mice in the P-407 + CAE group. CAE: Caerulein; SLC7A11: Solute carrier family 7 member 11; MDA: Malonaldehyde; GSH: Glutathione; GPX4: Glutathione peroxidase 4.

compared to P-407 + CAE group, while the MDA (Figure 4B) decreased to their normal range (all $P < 0.05$). The GSH level (Figure 4C) in the P-407 + CAE + Fer-1 group increased compared to P-407 + CAE group, while the Fe^{2+} (Figure 4D) decreased to their normal range (all $P < 0.05$). The histological scores of the pancreas, lung, and kidney in the P-407 + CAE + Fer-1 group were lower than those in the P-407 + CAE group (Figure 4E-G). The serum TNF-α, IL-1β, and IL-6 Levels in the P-407 + CAE + Fer-1 group were also lower than those in the P-407 + CAE group (all $P < 0.05$, Figure 4H). These results indicate that the inhibition of ferroptosis can attenuate the inflammatory response in mice with HTGP.

Inhibition of NADPH oxidase 2 can attenuate the inflammatory response in mice with HTGP through ferroptosis suppression

KEGG pathway analyses revealed overexpression of NADPH oxidase (NOX)2 in the enriched ferroptosis pathway in the P-407 + CAE group compared to the CAE group (Figure 5A and B). Next, we determined the role of NOX2 in regulating ferroptosis using Vas, an inhibitor of NOX2[7]. The expression of NOX2 was higher in the P-407 + CAE group than in the Ctrl group, while Vas significantly decreased the NOX2 expression level ($P < 0.05$, Figure 5C). In addition, MDA (Figure 5D) level decreased, GSH level increased (Figure 5E), Fe^{2+} (Figure 5F) level decreased and expression of GPX4 (Figure 5G) rebounded in the P-407 + CAE + Vas group compared to those in the P-407 + CAE group ($P < 0.05$). The histological scores of the pancreas, lung, and kidney in the P-407 + CAE + Vas group were lower than those in the P-407 + CAE group (all $P < 0.05$, Figure 5H-J). The serum TNF-α, IL-1β, and IL-6 Levels in the P-407 + CAE + Vas group were also lower than those in the P-407 + CAE group (all $P < 0.05$, Figure 5K). These results indicate that inhibition of NOX2 can attenuate the inflammatory response in mice with HTGP through attenuation of ferroptosis.

DISCUSSION

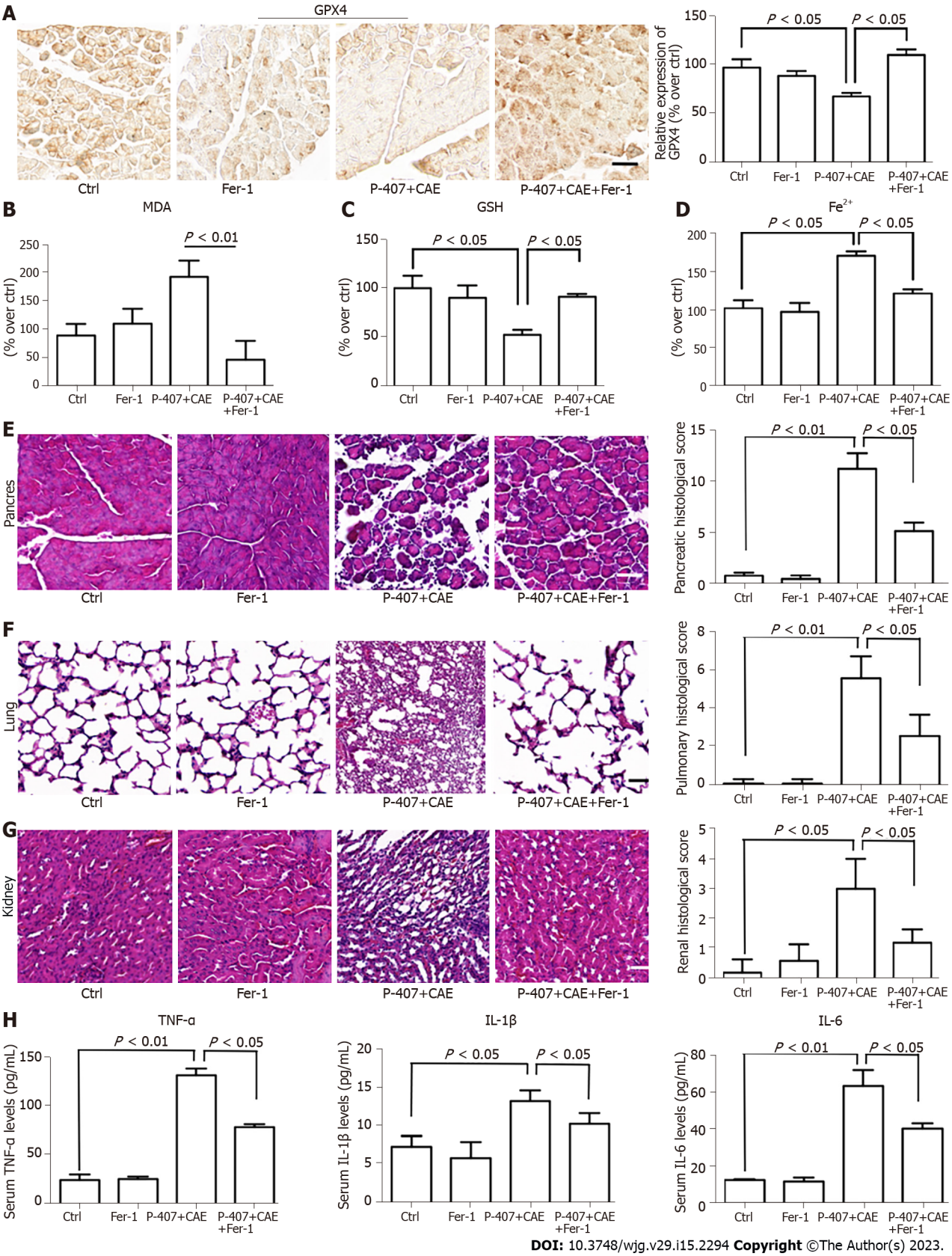
The early phase of HTGP is generally believed to be an unregulated inflammatory response or a cytokine storm that results in organ damage and death[31,32]. Pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, are considered pivotal mediators for initiating, amplifying, and perpetuating organ injury[33-35]. Therefore, timely blocking of the inflammatory response is considered critical for successful treatment.

As iron-dependent oxidative stress and lipid peroxidation are common features of ferroptosis and inflammatory diseases, ferroptosis, as a recognized form of RCD, has been reported to participate in the progression of inflammations[36]. Yet, its relationship with HTGP remains unclear. In the process of HTGP, abnormal lipid metabolism is also considered to have an important role, and FFA has been shown to induce the excessive release of ROS and accumulate in the body[4,5]. Previous studies have shown that the excessive production of ROS is a key factor for ferroptosis induction[22]. Therefore, we hypothesized that ferroptosis might also participate in the process of HTGP.

In this study, we found for the first time that ferroptosis has an important role in the process of HTGP, and its inhibition can attenuate the inflammatory response and improve prognosis in mice. To investigate these mechanisms, we induced a mouse model of HTGP by intraperitoneal injection of P-407 and CAE. Then, proteome sequencing predicted that ferroptosis is involved in the process of HTGP. To verify the results of proteome sequencing, we performed IHC staining of mouse pancreatic tissue. GSH, SLC7A11, and GPX4 expression levels were much lower in the P-407 + CAE group than in the AP group. Additionally, the MDA and Fe^{2+} level in the P-407 + CAE group was significantly higher than in the AP group. To further verify whether ferroptosis has a key role in regulating HTGP, we used Fer-1 to inhibit ferroptosis. The histological scores of pancreas and serum TNF-α, IL-1β, and IL-6 Levels in the P-407 + CAE + Fer-1 group were all significantly lower than those in the P-407 + CAE group.

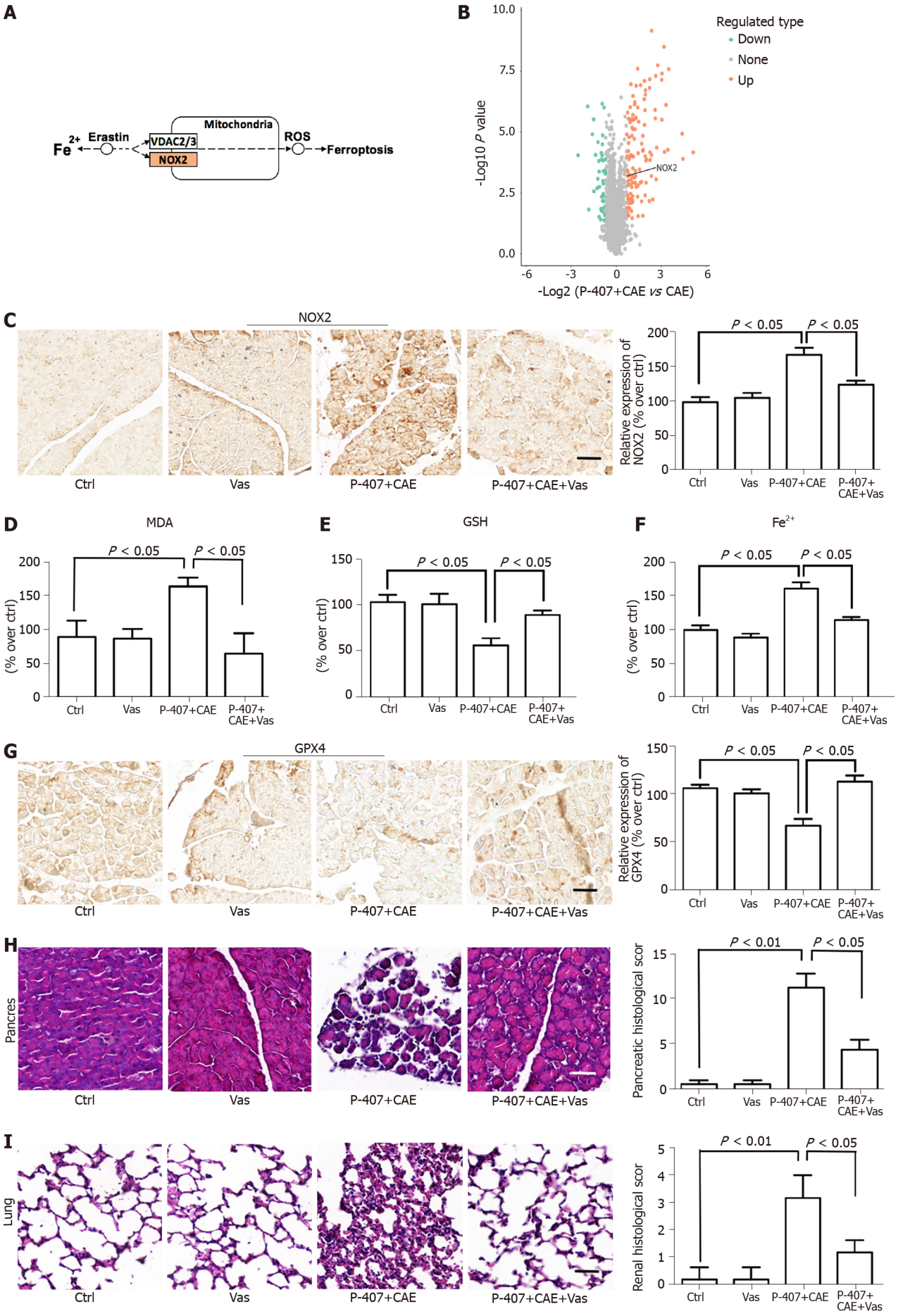
Ma *et al*[21] found that the inhibition of ferroptosis attenuated acute kidney injury in rats with severe AP (SAP), suggesting that ferroptosis may participate in SAP, but there was no distinction between the types of SAP[21]. Furthermore, our KEGG pathway analyses revealed that the upregulated proteins were strongly enriched for ferroptosis in the P-407 + CAE group compared to the CAE group, implying that ferroptosis occurs specifically in the P-407 + CAE group. Based on this finding, we speculate that in the process of HTGP, abnormal lipid metabolism produces excessive ROS, which induces ferroptosis and aggravates damage to the pancreas and other organs.

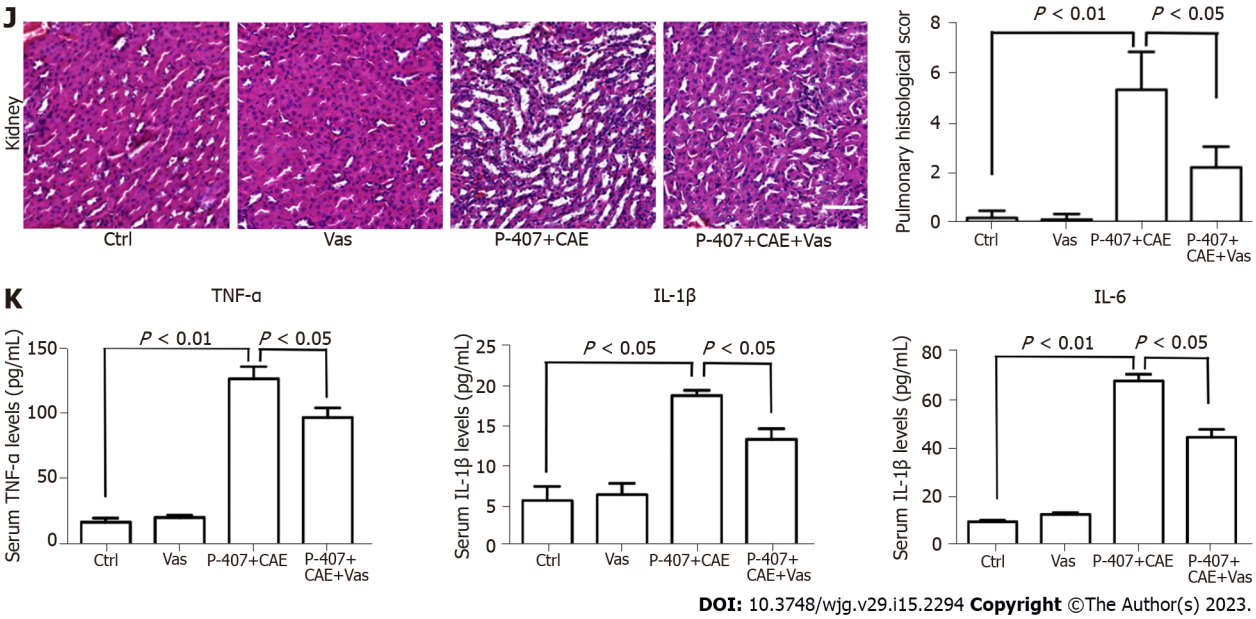
NOX is a ROS-generating transmembrane flavoprotein enzyme that is widely distributed in pancreas and kidney tissues[37,38]. Studies have shown that excessive ROS produced by NOX can activate the NF-κB pathway, aggravate cell death, and regulate inflammation[39]. Yang *et al*[40] suggested that NOX could have an important role in HTGP-associated kidney injury through the Akt/GSK-3β pathway[40]. Moreover, Wang *et al*[41] found that NOX2 is activated in an AMPK-dependent manner to promote oxidative stress in a diabetic rat model, which in turn led to ferroptosis and aggravated myocardial ischemia-reperfusion injury[41]. Our KEGG pathway analyses also revealed overexpression of NOX2 in the enriched ferroptosis pathway in in the P-407 + CAE group compared to the CAE group. Next, we verified whether NOX2 has a key role in the regulation of ferroptosis using Vas, an inhibitor of NOX2. We found that the serum MDA level decreased, and the expression of GPX4 in the P-407 + CAE + Vas



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Figure 4 The inhibition of ferroptosis attenuated P-407 + Caerulein-induced hypertriglyceridemic pancreatitis in mice (*n* = 4). A: Immunohistochemistry for Glutathione peroxidase 4 in the pancreas tissues of the P-407 + Caerulein (CAE) and P-407 + CAE + Ferrostatin-1 (Fer-1) groups; B: The malonaldehyde levels; C: The glutathione levels; D: Fe^{2+} levels in pancreases of P-407 + CAE and P-407 + CAE + Fer-1 groups; E: Hematoxylin–eosin (HE) staining and histological scores of the pancreas; F: HE staining and histological scores of the lung; G: HE staining and histological scores of the kidney; H: Serum tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 levels of the P-407 + CAE and P-407 + CAE + Fer-1 groups. NOX: NADPH oxidase; ROS: Reactive oxygen species; CAE: Caerulein; MDA: Malonaldehyde; Fer-1: Ferrostatin-1; GSH: Glutathione; GPX4: Glutathione peroxidase 4; TNF: Tumor necrosis factor; IL: Interleukin.


A
Fe 2+
Erastin
Mitochondria
VDAC2/3
NOX2
ROS
Ferroptosis
B
Regulated type
Down
None
Up
-Log10 P value
-Log2 (P-407+CAE vs CAE)
NOX2
C
NOX2
Ctrl
Vas
P-407+CAE
P-407+CAE+Vas
Relative expression of NOX2 (% over ctrl)
P < 0.05
P < 0.05
D
MDA
(% over ctrl)
E
GSH
F
Fe2+
G
GPX4
Relative expression of GPX4 (% over ctrl)
H
Pancres
Pancreatic histological scor
P < 0.01
P < 0.05
I
Lung
Renal histological scor



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Figure 5 The inhibition of NADPH oxidase 2 attenuated hypertriglyceridemic pancreatitis through a ferroptosis reduction in mice (*n* = 4). A: Kyoto Encyclopedia of Genes and Genomes pathways enriched with protein NADPH oxidase (NOX) 2 in comparison of P-407 + caerulein (CAE) group *vs* CAE group; B: Volcano plot showing the location of NOX2 in comparison of P-407 + CAE group *vs* CAE group; C: Immunohistochemistry (IHC) for NOX2 in the pancreas tissues of the P-407 + CAE and P-407 + CAE + Vas2870 (Vas) groups; D: The malonaldehyde levels; E: The glutathione levels; F: Fe^{2+} levels in the pancreases of the mice in the P-407 + CAE and P-407 + CAE + Vas groups; G: IHC for GPX4 in the pancreas tissues of the P-407 + CAE and P-407 + CAE + Vas groups; H: Hematoxylin–eosin (HE) staining and histological scores of the pancreas; I: HE staining and histological scores of the lung; J: HE staining and histological scores of the kidney; K: Serum TNF-α, IL-1β, and IL-6 levels of the P-407 + CAE and P-407 + CAE + Vas groups. The scale bar represents 50 mm. NOX: NADPH oxidase; ROS: Reactive oxygen species; CAE: Caerulein; Vas: Vas2870; MDA: Malonaldehyde; GSH: Glutathione; GPX4: Glutathione peroxidase 4; TNF: Tumor necrosis factor; IL: Interleukin.

group rebounded compared to the P-407 + CAE group. Meanwhile, the histological scores of the pancreas and the serum TNF-α, IL-1β, and IL-6 Levels in the P-407 + CAE + Vas group were significantly lower than those in the P-407 + CAE group. These results indicate that the inhibition of NOX2 attenuated HTGP through suppression of ferroptosis.

CONCLUSION

In summary, these data provide strong evidence that ferroptosis is involved in the process of HTGP and that NOX2 is a key point in the regulation of ferroptosis. The inhibition of ferroptosis and NOX2 attenuates the inflammatory response to improve patient outcomes. This discovery provides new insights into a better understanding of the pathogenesis of HTGP, which may be used as a novel therapeutic target in the future.

ARTICLE HIGHLIGHTS

Research background

Ferroptosis is involved in the development of inflammatory diseases, but its relationship with acute hypertriglyceridemic pancreatitis (HTGP) remains unclear.

Research motivation

HTGP has serious morbidity and high mortality. At present, there is no more effective method except symptomatic treatment. Therefore, exploring the pathogenesis of HTGP and seeking more accurate therapeutic targets are of critical importance.

Research objectives

This study aimed to explore whether ferroptosis is involved in the process of HTGP and elucidate its potential mechanisms in a mouse model.

Research methods

Pancreatic tissues from the model animals were subjected to proteome sequencing analysis. The pathological changes and scores of the pancreas, lung, and kidney were determined using hematoxylin–eosin staining. The levels of serum amylase, triglyceride, and total cholesterol were measured with an automatic blood cell analyzer. Additionally, the serum levels of tumor necrosis factor-α, interleukin (IL)-6, and IL-1β were determined by enzyme linked immunosorbent assay. Malonaldehyde, glutathione, and Fe^{2+} were detected in the pancreas. Finally, immunohistochemistry was performed to assess the expression of ferroptosis-related proteins.

Research results

Proteome sequencing revealed that ferroptosis was involved in the process of HTGP and that NADPH oxidase 2 may participate in ferroptosis regulation.

Research conclusions

Ferroptosis was found to have an important role in HTGP and may be considered a potential target for clinical treatment.

Research perspectives

This discovery provides new insights into a better understanding of the pathogenesis of HTGP, which may be used as a novel therapeutic target. However, the regulatory mechanism needs to be further explored in the future.

FOOTNOTES

Author contributions: Meng YT, Zhou Y, Han PY, and Ren HB designed the study; Meng YT, Zhou Y and Han PY participated in data collection and wrote the paper; Ren HB and Han PY were responsible for revising the manuscript, contributing the same to the paper; All the listed authors have contributed and approved the final manuscript.

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Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Case Control Study

Can visceral fat parameters based on computed tomography be used to predict occult peritoneal metastasis in gastric cancer?

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Abstract

BACKGROUND

The preoperative prediction of peritoneal metastasis (PM) in gastric cancer would prevent unnecessary surgery and promptly indicate an appropriate treatment plan.

AIM

To explore the predictive value of visceral fat (VF) parameters obtained from preoperative computed tomography (CT) images for occult PM and to develop an individualized model for predicting occult PM in patients with gastric carcinoma (GC).

METHODS

A total of 128 confirmed GC cases (84 male and 44 female patients) that underwent CT scans were analyzed and categorized into PM-positive (n = 43) and PM-negative (n = 85) groups. The clinical characteristics and VF parameters of two regions of interest (ROIs) were collected. Univariate and stratified analyses

based on VF volume were performed to screen for predictive characteristics for occult PM. Prediction models with and without VF parameters were established by multivariable logistic regression analysis.

RESULTS

The mean attenuations of $VF_{ROI\,1}$ and $VF_{ROI\,2}$ varied significantly between the PM-positive and PM-negative groups (P = 0.044 and 0.001, respectively). The areas under the receiver operating characteristic curves (AUCs) of $VF_{ROI\,1}$ and $VF_{ROI\,2}$ were 0.599 and 0.657, respectively. The mean attenuation of $VF_{ROI\,2}$ was included in the final prediction combined model, but not an independent risk factor of PM (P = 0.068). No significant difference was observed between the models with and without mean attenuation of VF (AUC: 0.749 *vs* 0.730, P = 0.339).

CONCLUSION

The mean attenuation of VF is a potential auxiliary parameter for predicting occult PM in patients with GC.

Key Words: Gastric carcinoma; Peritoneal metastasis; Visceral fat; Tomography; X-ray computed; Prediction; Individualized model

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Core Tip: The preoperative prediction of peritoneal metastasis (PM) in gastric cancer would prevent unnecessary surgery and promptly indicate an appropriate treatment plan. We therefore aimed to explore the predictive value of visceral fat (VF) parameters obtained from preoperative computed tomography images for occult PM and to develop an individualized model for predicting occult PM in patients with gastric carcinoma (GC). Consequently, mean attenuation of VF is a potential auxiliary parameter to predict occult PM in patients with GC.

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INTRODUCTION

Peritoneal metastasis (PM) is the major cause of postoperative distant metastasis and occurs in more than 10% of patients with newly diagnosed gastric cancer[1]. Gastric carcinoma (GC) is the third leading cause of cancer-related deaths worldwide. In gastric cancer with PM, the survival time is only a few months[2]. Many recent studies[3] have shown that the combination of complete cell reduction and intraperitoneal thermal perfusion chemotherapy can significantly prolong the median survival time in gastric cancer with PM. Thus, the preoperative prediction of PM in gastric cancer would prevent unnecessary surgery and promptly indicate an appropriate treatment plan. For example, laparoscopic exploration may be the preferred choice. Additionally, intraperitoneal hyperthermic chemotherapy may be prepared, and open exploration may be the final choice for a patient with occult PM.

Traditional preoperative imaging methods, such as computed tomography (CT) and magnetic resonance imaging, demonstrate high specificity and low sensitivity in the detection of PM[4]. Therefore, occult PM has attracted extensive attention; it refers to metastasis not indicated on preoperative CT imaging but detected during surgery and pathologic examination[5]. In the last few years, many studies have demonstrated the great potential of radiomics in the prediction of occult PM in gastric cancer[6]. However, most studies have used manual delineation of regions of interest (ROIs) in PM, which reduces repeatability and increases subjective dependence. Therefore, the urgent exploration for new parameters for predicting occult PM is warranted.

Tumor infiltration of the peritoneum alters the characteristics of the surrounding visceral fat (VF) on CT, and these changes have been investigated in previous radiomics studies[7]. In addition, the feature referred to as "smudge-like ground-glass opacity" in the abdominal cavity, which presents an increased CT attenuation of VF, is a common but easily overlooked sign of early-stage omental metastasis[8]. In addition, the adipose microenvironment has a subtle relationship with tumor progression[9].

We therefore aimed to explore the predictive value of VF parameters based on preoperative CT imaging for occult PM in GC and to develop an individualized model for predicting occult PM preoperatively.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the local institutional review board, and the requirement for informed consent was waived.

Patients at our hospital who met the inclusion/exclusion criteria were consecutively enrolled between July 2012 and July 2021. The inclusion criteria were as follows: (1) Diagnosis of gastric cancer by endoscopy and biopsy, with complete biopsy information; (2) Diagnosis of cT3 and cT4 gastric cancer by CT without obvious PM characteristics; and (3) PM status confirmed by exploratory surgery and pathological biopsy. The exclusion criteria were follows: (1) A history of abdominal tumors, cirrhosis, or other inflammatory diseases; (2) A history of abdominal surgery; (3) An interval between CT and surgical exploration longer than 2 wk; and (4) The presence of artifacts that affect the evaluation of VF parameters.

Clinical patient data (*e.g.*, sex and age), blood test information [*e.g.*, carcinoembryonic antigen (CEA), carbohydrate antigen 199 (CA199), and carbohydrate antigen 125 (CA125)], and biopsy information (*e.g.*, pathological type and differentiation) were collected for further analysis.

CT examination

CT examinations were performed using SOMATOM Definition Flash CT or SOMATOM Force (Siemens, Germany). All patients fasted for 6 h, and drank 800-1000 mL water 5-15 min before CT examination. Following an unenhanced scan, dual-phase enhanced CT images were obtained at 30- and 70-s delays. Approximately 1.3 mL/kg iodinated contrast agent (Ultravist 370, Bayer Schering Pharma) was injected intravenously at a rate of 3.0 mL/s. The parameters of the unenhanced scan were as follows: 120 kV tube voltage, 250–350 mAs, and 5 mm slice thickness and slice interval. The parameters of the enhanced scan were as follows: 120 kV tube voltage, automatic tube current modulation techniques, and 5- or 1.25-mm slice thickness and slice interval.

Fat assessment

The volume and mean CT attenuation of VF and subcutaneous fat (SF) were measured using unenhanced scan CT images on a workstation (MM Research Frontier SyngoVia, VB2.0, Siemens Healthineers, Forchheim, Germany). VF was defined as fat within the abdominal cavity, whereas SF was defined as fat below the skin. ROIs were manually drawn by tracing the medial edge of the abdominal muscle using a semiautomated technique, starting from the maximal axial section of the tumor and extending 15 mm (ROI 1) and 25 mm (ROI 2) downward. The contour of each layer can be adjusted manually as necessary. A primary radiologist (with 6 years of experience) was responsible for outlining, while another senior radiologist (with 25 years of experience) was responsible for supervising each operation (Figure 1). Twenty cases were randomly chosen for outlining by another radiologist (with 3 years of experience) to verify the consistency.

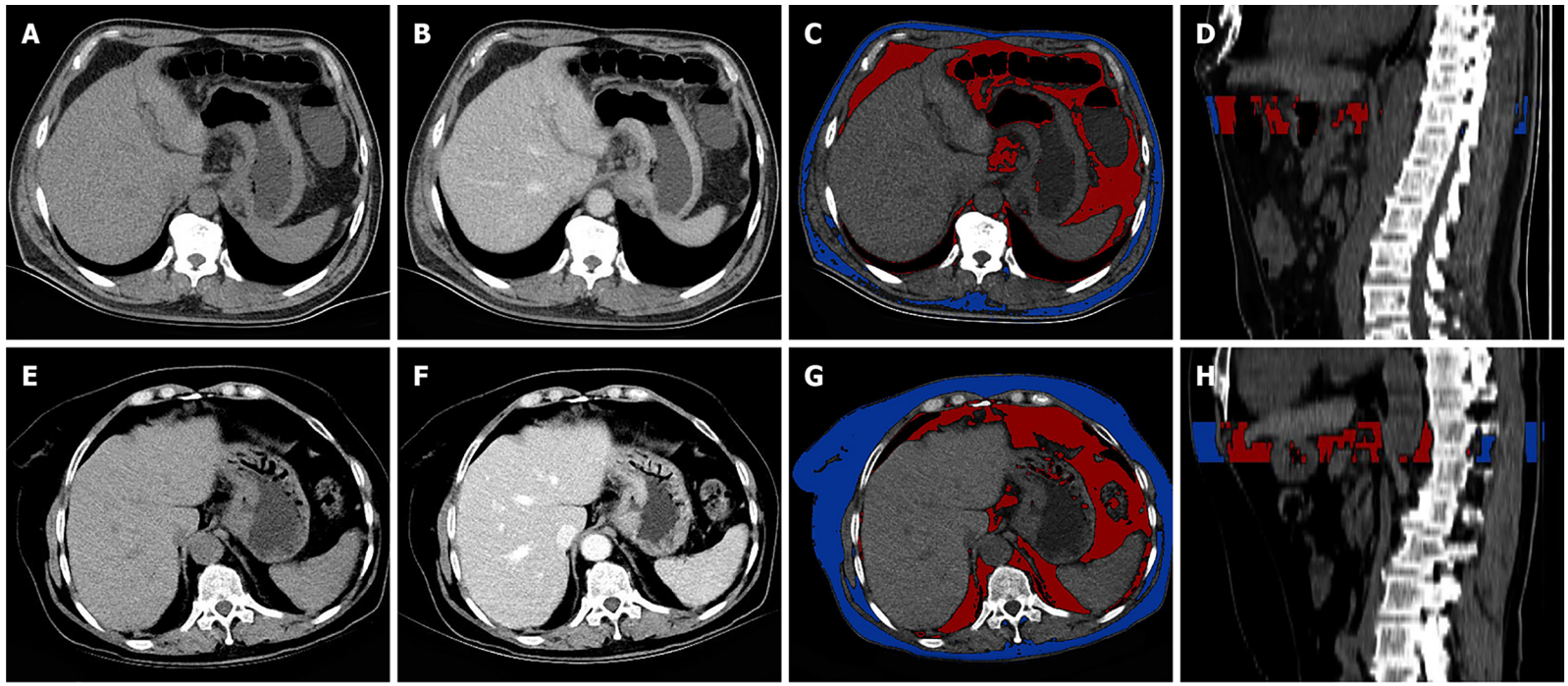
CT attenuation between −150 and −50 Hounsfield units (HU) was performed to evaluate voxels containing adipose tissue[10]. Adipose tissue volume was measured in cubic centimeters (cm^3), while mean CT attenuation was measured in HU. The mean CT attenuation and volume of VF were obtained directly on the workstation simultaneously with a table including the CT attenuation of each voxel. The 10^{th}, 30^{th}, 50^{th}, 70^{th}, 90^{th}, 25^{th}, and 75^{th} percentiles of CT attenuation were obtained during postprocessing and calculation.

Analysis of CT images

Two radiologists blinded to the pathologic data analyzed the CT characteristics. When disagreements occurred, a senior radiologist made the final decision. The cT and cN stages were analyzed according to the 8th American Joint Committee staging classification[11,12]. Mild ascites refers to ascites that could not be clearly determined to be caused by metastasis[5]. The GC thickness was measured at the maximal axial section[13].

Statistical analysis

Statistical analyses were conducted using SPSS software (version 22.0) and MedCalc software (version 15.2). Interobserver reliability was assessed using the intraclass correlation coefficient (ICC), where ICC ≥ 0.80 is excellent, 0.61-0.80 is good, and < 0.60 is the difference. The discrimination capability of various VF characteristics was evaluated using the receiver operating characteristic (ROC) curve and compared using Delong's test. The relationship between volume and mean attenuation was evaluated using Pearson correlation analysis. Stratified analysis based on VF volume was performed. The differences in



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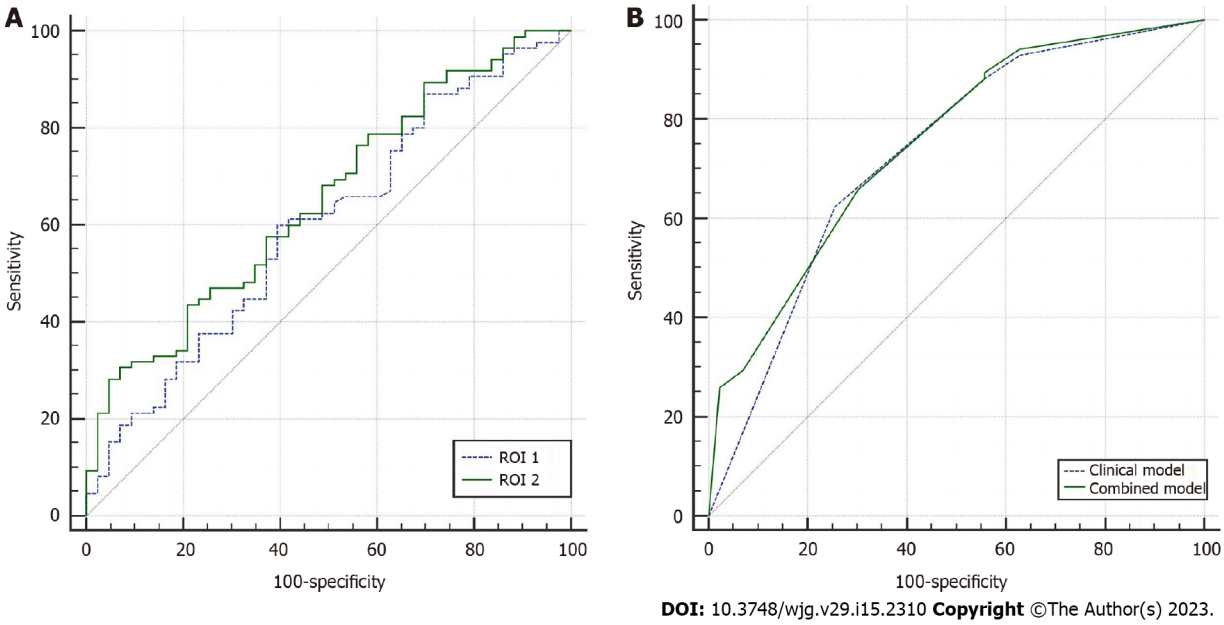
Figure 1 Determination of visceral fat parameters. A-D: Detection of visceral fat (VF) parameters in a 54-year-old male patient with gastric cancer with peritoneal metastasis (PM); A and B: Axial unenhanced and venous computed tomography (CT) images showing wall thickening in the cardia and the lesser and greater curvatures of the stomach; C: Regions of interest (ROI) were drawn using a semiautomated technique. The red area represents VF, while the blue area represents subcutaneous fat (SF); D: Sagittal image showing a three-dimensional (3D) ROI, delineated starting from the maximal axial section of the tumor and extending 25 mm; E-H: Determination of VF parameters in a 66-year-old female patient with gastric cancer without PM; E and F: Axial unenhanced and venous CT images showing irregular wall thickening and a mass in the cardia of the stomach; G and H: A 3D ROI drawn with a 25-mm height using a semiautomated technique. The red area represents VF, while the blue area represents SF.

continuous variables were compared using the two-tailed *t* test, Mann–Whitney *U* test, Kruskal-Wallis *H* test, and one-way ANOVA. The differences in categorical variables were compared using the χ^2 test. Significant variables were analyzed using multifactorial logistic regression analysis (backward: Conditional). $P < 0.05$ was considered to suggest a statistically significant difference.

RESULTS

Patient characteristics

A total of 43 PM-positive patients were included in the study, and 85 PM-negative patients treated



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Figure 2 Predictive performance of computed tomography attenuations for visceral fat in two regions of interest and two models for predicting peritoneal metastasis in gastric cancer. A: Receiving operator characteristic (ROC) curves of the visceral fat (VF) computed tomography (CT) attenuations on ROIs 1 and 2. The VF CT attenuations on ROI 2 [area under the curve (AUC): 0.657, sensitivity 93.0%, specificity 30.6%] showed a better predictive performance than those on ROI 1 (AUC: 0.599, sensitivity 60.0%, specificity 60.0%); B: ROC curves of the clinical and combined models. The two models showed comparable predictive performance for peritoneal metastasis in gastric cancer (AUC: 0.730 *vs* 0.749, $P = 0.399$)

between January 2021 and July 2021 were included as control subjects. Significant differences in age, CA125, tumor location, thickness, mild ascites, and cT ($P < 0.05$ for all) were found between the PM-positive and PM-negative groups (Table 1). No significant differences were found for sex, pathological type, differentiation, CEA, CA199, or cN stage ($P > 0.05$ for all).

VF characteristics

Significant differences in the mean attenuations of $VF_{ROI\,1}$ and $VF_{ROI\,2}$ ($P = 0.044$ and 0.001, respectively) were found between the PM-positive and PM-negative groups (Table 2). No significant differences were found for the mean attenuations of $SF_{ROI\,1}$ and $SF_{ROI\,2}$, the volumes of $VF_{ROI\,1}$, $VF_{ROI\,2}$, $SF_{ROI\,1}$, and $SF_{ROI\,2}$, or the volume ratio VF/SF ($P > 0.05$ for all).

The areas under the receiver operating characteristic curves (AUCs) of $VF_{ROI\,1}$ and $VF_{ROI\,2}$ for predicting PM in GC were 0.599 [95% confidence interval (CI): 0.509-0.685] and 0.657 (95%CI: 0.568-0.738), respectively (Figure 2A). The comparison of the ROC curves of the VF characteristics suggested that $VF_{ROI\,2}$ significantly outperformed $VF_{ROI\,1}$ ($P = 0.002$). Most of the interobserver agreements between the two operators were excellent (Table 2).

Significant differences in the 30th, 50th, 70th, 25th, and 75th percentiles of CT attenuation of $VF_{ROI\,2}$ were found between the PM-positive and PM-negative groups (Supplementary Table 1). The AUCs of the 10th, 30th, 50th, 70th, 90th, 25th, and 75th percentiles of CT attenuation for predicting PM in GC were 0.612, 0.642, 0.641, 0.619, 0.608, 0.629, and 0.619, respectively. The comparison of ROC curves suggested no significant difference between the 30th percentile and the mean CT attenuation of $VF_{ROI\,2}$ ($P = 0.664$) (Supplementary Figure 1).

Relationship between VF and clinical characteristics

Significant differences in the mean attenuation of $VF_{ROI\,2}$ were observed between the different cN and cT stage classifications ($P = 0.030$ and 0.003, respectively). The mean attenuation of $VF_{ROI\,2}$ in patients with elevated CA125, elevated CA199, and mild ascites was significantly higher than that in normal conditions ($P = 0.002$, 0.039, and 0.001, respectively) (Table 3).

Stratified analysis

The mean attenuation was negatively correlated with the volume of $VF_{ROI\,2}$ (Pearson correlation coefficient = -0.688; $P = 0.001$) (Supplementary Figure 2). Patients were divided into two groups according to the median volume of $VF_{ROI\,2}$ (median = 141.58 cm^3). Stratified analysis suggested that the mean attenuation of $VF_{ROI\,2}$ was more effective in predicting occult PM in the high-volume group than in the low-volume group (Table 4, Figure 3).

Table 1 Comparison of clinical and computed tomography characteristics between two groups, *n* (%)

Variable		PM (-) (*n* = 85)	PM (+) (*n* = 43)	*P* value
Sex	Male	60 (70.6)	24 (55.8)	0.096
	Female	25 (29.4)	19 (44.2)	
Age (yr) (mean ± SD)		56.44±12.63	61.25±11.11	0.029[1]
Pathological type	Adenocarcinoma	83 (97.6)	38 (88.4)	0.077
	Non-adenocarcinoma	2 (2.4)	5 (11.6)	
Differentiation	Poorly differentiated	50 (58.8)	30 (69.8)	0.227
	Moderately and well differentiated	35 (41.2)	13 (30.2)	
CEA	Normal	74 (87.1)	37 (86.0)	0.873
	Elevated	11 (12.9)	6 (14.0)	
CA199	Normal	74 (87.1)	32 (74.4)	0.073
	Elevated	11 (12.9)	11 (25.6)	
CA125	Normal	83 (97.6)	37 (86.0)	0.030[1]
	Elevated	2 (2.4)	6 (14.0)	
Location	Cardia	44 (51.8)	7 (16.3)	0.001[1]
	Body	12 (14.1)	14 (32.6)	
	Antrum	20 (23.5)	13 (30.2)	
	≥ 2 parts	9 (10.6)	9 (20.9)	
Mild ascites	(-)	75 (88.2)	24 (55.8)	0.001[1]
	(+)	10 (11.8)	29 (44.2)	
cN stage	N0	26 (30.6)	11 (25.6)	0.150
	N1-2	41 (48.2)	16 (37.2)	
	N3-4	18 (21.2)	16 (37.2)	
cT stage	≤ T3	57 (67.1)	14 (32.6)	0.001[1]
	≥ T4	28 (32.9)	29 (67.4)	
Thickness (mm), median (IQR)		15.11 (13.02, 18.85)	13.43 (11.28, 17.83)	0.023[1]

[1]$P < 0.05$.

PM: Peritoneal metastasis; CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen 199; CA125: Carbohydrate antigen 199; IQR: Interquartile range.

Risk factors for occult PM

Multiple logistic regression analysis based on clinical characteristics showed that mild ascites and cT stage (≥ T4) were independent risk factors for occult PM (P = 0.003 and 0.010, respectively) (Table 5). The Hosmer-Lemeshow test revealed that the model had a good fit (P = 0.977). Furthermore, the clinical model showed a moderate prediction performance (AUC = 0.730, 95%CI: 0.644-0.804).

Multiple logistic regression analyses of both clinical characteristics and VF parameters showed that the mean attenuation of $VF_{ROI\,2}$, mild ascites, and cT stage (≥ T_4) were included in the final prediction combined model, but the mean attenuation of $VF_{ROI\,2}$ was not an independent risk factor for PM (P = 0.068) (Table 5). The Hosmer-Lemeshow test indicated that the model had a good fit (P = 0.523). The combined model showed a moderate prediction performance (AUC = 0.749, 95%CI: 0.665-0.821). No significant difference was found between the prediction performance of the two models (P = 0.339) (Figure 2B).

DISCUSSION

We explored the value of VF parameters in predicting occult PM in GC and subsequently developed a preoperative prediction model based on clinical features and VF parameters. A significant difference in the mean attenuation of VF was found between the PM-positive and PM-negative groups. The mean

Table 2 Comparison of visceral fat characteristics between the two lesions

Variable	PM (+) (*n* = 43)	PM (-) (*n* = 85)	*P* value	ICC
$VF_{ROI\ 1}$				
Volume (cc)	84.45 (36.70, 161.04)	87.92 (49.89, 160.75)	0.412	0.913
Mean attenuation (HU)	-82.07 ± 6.06	-85.33 ± 5.89	0.044[1]	0.810
SD attenuation (HU)	18.82 (17.78, 21.25)	19.51 (18.63, 21.11)	0.133	0.926
$SF_{ROI\ 1}$				
Volume (cc)	108.79 (55.10, 170.90)	115.96 (62.70, 178.72)	0.760	0.979
Mean attenuation (HU)	-85.33 ± 5.89	-86.05 ± 8.23	0.934	0.955
SD attenuation (HU)	17.94 (16.60, 19.37)	18.58 (17.27, 20.16)	0.180	0.981
$VF_{ROI\ 2}$				
Volume (cc)	132.63 (58.85, 273.65)	145.48 (82.58, 277.42)	0.302	0.932
Mean attenuation (HU)	-82.19 ± 5.35	-85.70 ± 5.65	0.001[1]	0.974
SD attenuation (HU)	19.86 (17.58, 20.46)	19.13 (18.47, 20.66)	0.317	0.950
$SF_{ROI\ 2}$				
Volume (cc)	163.24 (82.57, 261.17)	175.86 (97.86, 267.52)	0.76	0.991
Mean attenuation (HU)	-86.28 ± 8.61	-86.41 ± 8.08	0.938	0.980
SD attenuation (HU)	17.80 (16.42, 19.33)	18.45 (17.29, 19.91)	0.124	0.993
Volume ratio				
$VF/SF_{ROI\ 1}$	0.92 (0.44, 1.50)	1.00 (0.61, 1.65)	0.317	0.804
$VF/SF_{ROI\ 2}$	0.98 (0.47, 1.41)	1.00 (0.63, 1.68)	0.279	0.884

[1]$P < 0.05$.

PM: Peritoneal metastasis; ICC: Intraclass correlation coefficient; VF: Visceral fat; SF: Subcutaneous fat; SD: Standard deviation; ROI: Region of interest; HU: Hounsfield units; ROIs 1 and 2: Regions of interest starting at the largest axial level of the tumor and extending down 15 mm and 25 mm, respectively.

attenuation of VF was included in the final prediction combined model but was not an independent predictor of occult PM in GC.

Previous studies on VF have mostly focused on the effect of volume or area on postoperative prognosis, and few studies have investigated the CT attenuation of VF[14,15]. CT attenuation of pericoronary adipose tissue has been shown to be strongly correlated with cardiovascular disease[16]. Xiang *et al*[9] investigated the relationship between adipocytes and gastric cancer cells and found that adipocytes may promote cancer cell invasion through a specific signaling pathway. CT attenuation is an index reflecting changes in the VF microenvironment, and its increase may be closely related to tumor progression[17]. Given all of that, we believe that certain changes occur in VF with PM. The indicators used to quantify such changes in our study included the mean attenuation, volume, and volume ratio. Previous studies[5,18] have suggested that the radiomics features of the adjacent peritoneum are helpful in predicting PM in GC. Therefore, two nearby ROIs that delineated from the maximal axial section of the tumor were adopted in our study, and these ROIs were considered to be the most likely sites of metastasis.

The regression analysis indicated that the mean attenuation of $VF_{ROI\ 2}$ was not an independent risk factor for PM, and it had a lower predictive ability than ascites and cT stage. The changes in the adipose microenvironment and the increase in the mean attenuation of VF may be mainly concentrated around the tumor, and the ROI of VF on the whole axial level may dilute this change and reduce the predictive sensitivity. However, the results confirmed that the mean attenuation of VF was significantly different between the PM-positive and PM-negative groups in both ROI 1 and ROI 2. The 30^{th}, 50^{th}, 70^{th}, 25^{th}, and 75^{th} percentiles of attenuation of $VF_{ROI\ 2}$ were also significantly different between the two groups. Moreover, the mean attenuation of VF was related to clinical features representing tumor invasiveness. Therefore, we have reasons to believe that the mean attenuation of VF is a potential auxiliary parameter for predicting occult PM in patients with GC, especially for those with only nonenhanced CT images or upper abdominal CT images.

The mean attenuation was negatively correlated with the volume of VF; however, noticeably, no significant difference in the VF volume was found between the PM-positive and PM-negative groups. Considering the relationship between the mean attenuation and volume of VF, volume-stratified

Table 3 Comparison of attenuations of visceral fat between different clinical characteristic classifications

Variable		Mean attenuation of VF ROI 2	*P* value
Sex	Male	-84.90 ± 6.19	0.306
	Female	-83.83 ± 4.88	
Age (yr)	≤ 61	-84.48 ± 5.11	0.937
	> 61	-84.46 ± 5.14	
Pathological type	Adenocarcinoma	-84.64 ± 5.81	0.339
	Non-adenocarcinoma	-82.49 ± 4.99	
Differentiation	Poorly differentiated	-84.18 ± 5.91	0.377
	Moderately and well differentiated	-85.11 ± 5.56	
CEA	Normal	-84.64 ± 5.92	0.583
	Elevated	-83.81 ± 4.86	
CA199	Normal	-85.04 ± 6.04	0.002[1]
	Elevated	-82.07 ± 3.40	
CA125	Normal	-84.80 ± 5.81	0.039[1]
	Elevated	-80.46 ± 3.41	
Location	Cardia/Body/Antrum	-84.93 ± 5.86	0.049[1]
	≥ 2 parts	-82.05 ± 4.67	
Mild ascites	(-)	-85.41 ± 5.79	0.001[1]
	(+)	-81.52 ± 4.69	
cN stage	N0	-85.48 (-89.85, -82.95)	0.030[1]
	N1-2	-83.08 (-86.68, -79.43)	
	N3-4	-84.54 (-87.51, -80.60)	
cT stage	≤ T3	-85.88 ± 5.83	0.003[1]
	≥ T4	-82.84 ± 5.29	
Thickness (mm)	≤ 14.5	-84.82 ± 6.36	0.517
	> 14.5	-84.15 ± 4.98	

[1]$P < 0.05$.

CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen 199; CA125: Carbohydrate antigen 199; VF: Visceral fat; ROI 2: Regions of interest starting at the largest axial level of the tumor and extending down 25 mm.

Table 4 Stratified analysis of volume

	PM (-)	PM (+)	*P* value	AUC (95%CI)
Volume > median				
No	45	19		
Mean attenuation (HU)	-89.08 ± 5.31	-85.44 ± 3.93	0.009[1]	0.726 (0.59-0.854)
Volume ≤ median				
No	40	24		
Mean attenuation (HU)	-81.92 ± 3.01	-79.61 ± 4.94	0.046[1]	0.639 (0.490-0.787)

[1]$P < 0.05$.

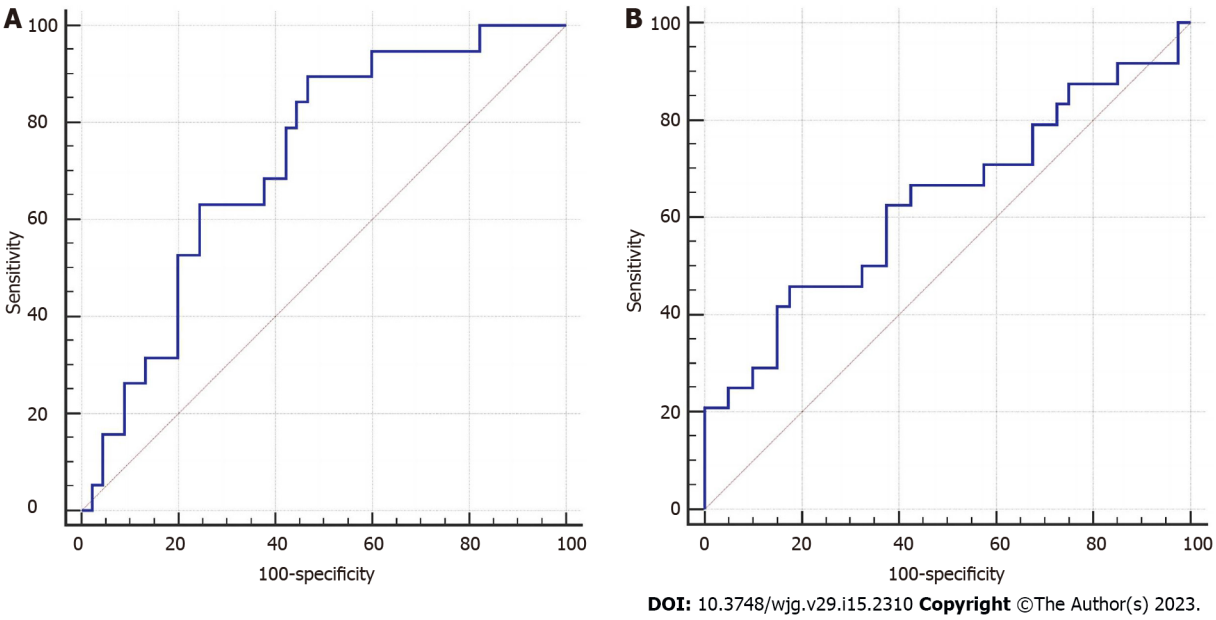
PM: Peritoneal metastasis; No: Number; AUC: Area under the curve; HU: Hounsfield units.

Table 5 Variables and coefficients of multiple regression logistic analysis

Variable	OR	95%CI	*P* value
Based on all characteristics			
Mild ascites (+ *vs* -)	3.552	(1.375-9.175)	0.009[1]
Mean attenuations of $VF_{ROI\,2}$	3.437	(0.914-12.914)	0.068
cT stage (≥ T4 *vs* ≤ T3)	2.631	(1.126-6.144)	0.025[1]
Intercept	0.009		0.001[1]
Based on clinical characteristics			
Mild ascites (+ *vs* -)	4.188	(1.636-10.722)	0.003[1]
cT stage (≥ T4 *vs* ≤ T3)	2.992	(1.301-6.881)	0.010[1]
Intercept	0.068		0.001[1]

[1]$P < 0.05$.

VF: Visceral fat; ROI: Region of interest; OR: Odds ratio; CI: Confidence interval.



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Figure 3 Performance of computed tomography attenuations for visceral fat in predicting peritoneal metastasis in gastric cancer. A: High-volume group; B: Low-volume group.

analysis was applied to minimize the deviations in volume variation. The mean attenuation of $VF_{ROI\,2}$ showed a higher predictive potential in the high-volume group than in the low-volume group. This result suggests that patients with high-volume VF may have more hypoxic or disordered adipocytes that secrete proinflammatory cytokines and angiogenic factors and promote tumor metastasis than patients with low-volume VF[19]. The attenuation of VF was significantly correlated with cT stage, cN stage, CA125, and CA199, indicating that increased VF attenuation is highly correlated with tumor progression.

Mild ascites and cT stage are independent predictive factors for occult PM. Liu *et al*[7] reported that cT stage was significantly correlated with occult PM, which reflects tumor aggressiveness. Mild ascites was also incorporated into a clinical model for predicting occult PM in a multicenter study[5]. Significant differences were observed in the mean attenuations of VF between the different ascites statuses and cT stages.

Compared with previous radiomics studies, the extraction of VF parameters in our study was based on unenhanced CT images through semiautomated sketching technology, which would reduce the selection differences between operators. The consistency between the operators of VF parameters was excellent. The ROIs in the peritoneum used in most previous studies on occult PM were manually sketched[5,18]. Thus, evaluating the consistency among different operators in defining the location and area of ROIs selected was difficult. In addition, three-dimensional ROIs were defined in our study,

which remain applicable in patients with lesser VF.

This study has certain limitations. First, the VF parameters were measured using data obtained from a single center with a small sample size, and multicenter studies with a large sample size are thus needed to validate the results. Second, the parameters used to quantify VF changes were obtained only from unenhanced CT images, which warrants the need to consider more parameters from multiphase enhanced images.

CONCLUSION

In conclusion, our study demonstrates the great potential of VF parameters in predicting occult PM in GC and presents a noninvasive preoperative model that combines the mean attenuation of VF and clinical factors for predicting occult PM in GC.

ARTICLE HIGHLIGHTS

Research background

The preoperative prediction of peritoneal metastasis (PM) in gastric cancer (GC) would prevent unnecessary surgery and promptly indicate an appropriate treatment plan.

Research motivation

Tumor infiltration of the peritoneum alters the characteristics of the surrounding VF on computed tomography (CT), and these changes have been investigated in previous studies.

Research objectives

We therefore aimed to explore the predictive value of VF parameters obtained from preoperative CT images for occult PM and to develop an individualized model for predicting occult PM in patients with GC.

Research methods

A total of 128 confirmed GC cases that underwent CT scans were analyzed and categorized into PM-positive and PM-negative groups. The clinical characteristics and VF parameters of two regions of interest (ROIs) were collected. Univariate and stratified analyses based on VF volume were performed to screen for predictive characteristics for occult PM. Prediction models with and without VF parameters were established by multivariable logistic regression analysis.

Research results

The mean attenuations of $VF_{ROI\,1}$ and $VF_{ROI\,2}$ varied significantly between the PM-positive and PM-negative groups (P = 0.044 and 0.001, respectively). The mean attenuation of $VF_{ROI\,2}$ was included in the final prediction combined model, but not an independent risk factor of PM (P = 0.068). No significant difference was observed between the models with and without mean attenuation of VF (area under the curve: 0.749 *vs* 0.730, P = 0.339).

Research conclusions

The mean attenuation of VF is a potential auxiliary parameter for predicting occult PM in patients with GC.

Research perspectives

Our study demonstrates the great potential of VF parameters in predicting occult PM in GC and presents a noninvasive preoperative model that combines the mean attenuation of VF and clinical factors for predicting occult PM in GC.

FOOTNOTES

Author contributions: Li LM, Huang WP, and Gao JB designed the research study; Huang WP, Li LM, and Gao JB performed the research; Yu Y and Cheng PY contributed analytic tools; Li LM, Feng LY, and Liu CC analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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ORIGINAL ARTICLE

Retrospective Study

Value of red blood cell distribution width in prediction of diastolic dysfunction in cirrhotic cardiomyopathy

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Abstract

BACKGROUND

Clinical diagnosis of cirrhotic cardiomyopathy (CCM) often encounters challenges of lack of timeliness and disease severity, with the commonly positive indicator usually associated with advanced heart failure.

AIM

To explore suitable biomarkers for early CCM prediction.

METHODS

A total of 505 eligible patients were enrolled in this study and divided into four groups according to Child-Pugh classification: Group I, Class A without CCM (105 cases); Group II, Class A with CCM (175 cases); Group III, Class B with CCM (139 cases); and Group IV, Class C with CCM (86 cases). Logistic regression and receiver operating characteristic (ROC) curve analyses were performed to determine whether red blood cell distribution width (RDW) was an independent risk factor for CCM risk. The relationships between RDW and Child-Pugh scores, Model for End-Stage Liver Disease (MELD) scores, and N-terminal pro-brain natriuretic peptide (NT-proBNP) were analyzed by Pearson correlation analysis.

RESULTS

A constant RDW increase was evident from Group I to Group IV (12.54 ± 0.85, 13.29 ± 1.19, 14.30 ± 1.96, and 16.25 ± 2.13, respectively). Pearson correlation analysis showed that RDW was positively correlated with Child-Pugh scores (r = 0.642, P < 0.001), MELD scores (r = 0.592, P < 0.001), and NT-proBNP (r = 0.715, P < 0.001). Furthermore, between Group I and Group II, RDW was the only significant index (odds ratio: 2.175, 95% confidence interval [CI]: 1.549-3.054, P < 0.001), and it reached statistical significance when examined by ROC curve

analysis (area under the curve: 0.686, 95%CI: 0.624-0.748, $P < 0.001$).

CONCLUSION
RDW can serve as an effective and accessible clinical indicator for the prediction of diastolic dysfunction in CCM, in which a numerical value of more than 13.05% may indicate an increasing CCM risk.

Key Words: Cirrhotic cardiomyopathy; Child-Pugh; Diagnosis; N-terminal pro-brain natriuretic peptide; Red blood cell distribution width

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Core Tip: Diastolic dysfunction is usually an early stage of cirrhotic cardiomyopathy (CCM). This study found that red blood cell distribution width would have an advantage over N-Terminal pro-brain natriuretic peptide in indicating diastolic dysfunction in such patients, which is of great significance for the early diagnosis and treatment of CCM.

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INTRODUCTION

As a manifestation of unnoticeable cardiac dysfunction, cirrhotic cardiomyopathy (CCM) features diastolic dysfunction, chronotropic dysfunction, electrophysiological abnormalities, and compromised myocardial contractility in the absence of other cardiac diseases. In such a setting, liver cirrhosis-induced hyperdynamic circulation may even result in structural cardiac abnormalities[1,2]. Available literature evidences that CCM-associated diastolic dysfunction can pose very adverse clinical implications[3], including severe liver diseases, hepatorenal syndrome, ascites[4,5], and death[6]. The current clinical diagnosis of CCM-related diastolic dysfunction mainly relies on transthoracic echocardiography (TTE)[7], with N-terminal pro-brain natriuretic peptide (NT-proBNP) and other indicators playing a supportive role[8,9]. However, studies contend that NT-proBNP level is not an optimal biomarker for CCM screening and cannot serve as an independent diagnostic tool for such a condition, though increased NT-proBNP level in liver cirrhosis has been found to be positively associated with echocardiographic measures of left ventricular diastolic dysfunction (LVDD) and may be indicative of CCM[10]. Therefore, an urgent need remains regarding a more objective indicator for CCM screening in patients with LVDD, especially in the early stages of the disease.

Of all the other potential candidate indicators, red blood cell distribution width (RDW), a common blood laboratory parameter, has been closely associated with cardiac function and/or the disease severity of heart failure (HF), including the natriuretic peptides[11], left ventricular end diastolic pressure[12], and left ventricular deformation[13,14]. Meanwhile, RDW levels are more or less correlated with various liver diseases[15-18]. Studies have demonstrated that the assessment of RDW may improve risk stratification of patients with acute decompensation of cirrhosis[19]. Other studies have documented RDW as a novel inflammatory marker in various conditions, including cardiovascular diseases[20], functional bowel conditions[21], autoimmune diseases[22,23], degenerative vertebral conditions[24], malignancy[25], and even COVID-19[26]. Taken together, these findings evidence that RDW may be altered in cirrhosis patients comorbid with cardiac diastolic dysfunction and may serve as a promising objective indicator for CCM screening. Therefore, this study attempted to probe into the value of RDW in the prediction of LVDD in CCM patients.

MATERIALS AND METHODS

Patient recruitment

A single-center, retrospective study was designed to evaluate the correlation between RDW levels and liver cirrhosis with diastolic dysfunction. The clinical data of 5205 patients diagnosed with cirrhosis of various etiologies were collected between June 2017 and May 2022, and a total of 505 cases were selected and included in this study. All these patients were referred to Fujian Medical University Union Hospital

(Fuzhou, Fujian Province, China). For patients hospitalized more than once, data from the first admission were used. The following items were set to support a diagnosis of liver cirrhosis[27,28]: (1) Imaging by ultrasonography, computed tomography, or magnetic resonance imaging indicating irregular liver surface and heterogeneous liver parenchyma; (2) impaired liver synthetic function: Albumin level less than 35.0 g/L without other identifiable causes of hypoalbuminemia such as renal loss or gastrointestinal loss; and (3) evidence of portal hypertension (variceal hemorrhage, refractory ascites, or splenomegaly) or life-threatening complications such as spontaneous bacterial peritonitis or hepatic encephalopathy. Liver cirrhosis was defined as conditions meeting criteria (1) and (2) or (1) and (3).

The inclusion criteria were: (1) Age over 18 years; (2) diagnosis of cirrhosis confirmed as above mentioned; (3) severity of liver cirrhosis evaluated by Child-Pugh classification and Model for End-Stage Liver Disease (MELD) score; and (4) LVDD confirmed by TTE (controls without LVDD). The exclusion criteria were: (1) Known cardiac diseases (such as valvular heart disease, rhythm or conduction disorders, congenital heart disease, coronary artery disease, non-ischemic cardiopathy, and pulmonary arterial hypertension); (2) TTE-confirmed systolic dysfunction only; (3) other causes of liver insufficiency (such as septicaemia and toxic liver disease); (4) RDW increase-related pre-existing conditions such as thalassemia, hemolytic anemia, hereditary spherocytosis, sickle cell disease, myelodysplastic syndrome, or aplastic anemia; and (5) other underlying conditions including gastric or duodenal ulcer, pregnancy, active malignancy other than liver cancer, and recent transfusion or use of iron or erythropoietin (within past 3 mo).

In particular, this study not only explored the changes of RDW in patients with CCM, but more importantly, revealed the value of RDW in predicting early CCM by comparing the differences of RDW in cirrhotic patients with or without cardiomyopathy. As a result, we set a group consisting of Child-Pugh A cirrhotic patients without CCM as the control group.

Measurements of baseline variables

Upon admission, blood samples were collected at 6 a.m. and processed immediately at the clinical laboratory of Fujian Medical University Union Hospital. The reference range of RDW was 11.5%-15.0%. Cardiac function was measured by TTE (Philips iE33, Philips Medical Systems, Andover, MA, United States). According to the latest CCM guidelines [2019 Cirrhotic Cardiomyopathy Consortium (CCC)][7], LVDD in CCM was diagnosed when three of the following conditions were present: E/e' ≥ 15 [early diastolic transmitral and myocardial velocity on Doppler tissue imaging ratio], peak tricuspid regurgitation velocity > 2.8 m/s, septal e' velocity < 7 cm/s (early diastolic myocardial velocity on TDI), and left atrial volume index > 34 mL/m^2.

Spontaneous bacterial peritonitis (SBP) was defined as a bacterial infection of the ascitic fluid in the absence of a secondary intra-abdominal focus and determined by ascitic fluid polymorphonuclear (PMN) count, in which PMN count ≥ 250 cells/mm^3 was diagnosed as SBP[29]. Refractory ascites was defined as ascites that cannot be mobilized or the early recurrence of which (after a large volume paracentesis) cannot be prevented by medical therapy[30]. At last, hepatic encephalopathy (HE) was defined as brain dysfunction in patients with liver failure and/or portosystemic shunts presenting cognitive alterations and personality and mental disorders[31].

Statistical analysis

The data were analyzed with SPSS version 26.0 (IBM, Somers, NY, United States). The normality of the data was evaluated by Kolmogorov-Smirnov test. For normally-distributed continuous variables, the data are presented as the mean ± SD; inter-group comparison was performed by one-way analysis of variance (ANOVA) and pairwise comparison by least significant difference *T* test (LSD-T). For abnormally-distributed continuous variables, data are presented as the median (interquartile range); inter-group and pairwise comparisons were analyzed by Kruskal-Wallis test and Bonferroni correction, respectively. The count data are presented as percentages (%). The proportions were measured by Chi-square test and Fisher's exact test was applied if the expected frequency was < 5. The potential association between RDW and CCM was examined by multivariate logistic regression analysis. The discriminatory power was evaluated by the area under the receiver operating characteristic (ROC) curve (AUC) with its 95% confidence interval (CI). The statistical significance was set at a two-sided probability value of $P < 0.05$. Simultaneously, Pearson correlation analysis was used to compare the relationships between RDW and Child-Pugh scores, MELD scores, and NT-proBNP.

RESULTS

Demographic features

In addition to the 400 cases diagnosed with CCM, we matched 105 patients with early cirrhosis without cardiomyopathy. According to Child-Pugh classification[9], all 505 eligible patients were divided into four groups: Group I, Class A without CCM (control group); Group II, Class A with CCM; Group III, Class B with CCM; Group IV, Class C with CCM. The baseline demographic and clinical characteristics

of the patient cohort are shown in Table 1. Patients with more serious Child-Pugh rating featured a significantly high incidence rate of hyperlipidemia and atrial fibrillation, though no statistical significance was found for age, gender, body mass index, smokers, and other demographic information among the groups.

Clinical information

The clinical symptoms, laboratory parameters, and medications of the enrolled patients are summarized in Table 2. Generally, with the worsening disease severity, more obvious complications were evident from Group I to Group IV, such as haematemesis (5.7%, 6.9%, 11.5%, and 18.6%, respectively, $P = 0.009$), edema (1.9%, 6.9%, 14.4%, and 38.4%, respectively, $P < 0.001$), SBP (1%, 2.3%, 7.9%, and 15.1%, respectively, $P < 0.001$), hyponatremia (2.9%, 12%, 22.3%, and 36%, respectively, $P < 0.001$), HE (4.8%, 5.7%, 10.1%, and 22.1%, respectively, $P < 0.001$), refractory ascites (1.9%, 2.3%, 10.8%, and 24.4%, respectively, $P < 0.001$), and jaundice (11.4%, 7.4%, 23.3%, and 24.4%, respectively, $P = 0.003$). Along with the presence of these indicators, MELD score also increased in the four groups (8.74 ± 1.54, 8.64 ± 1.57, 17.04 ± 1.96, and 25.19 ± 2.15, respectively, $P < 0.001$). Except for alkaline phosphatase (ALP), regardless of the increase or decrease along with the rise of Child-Pugh level, a statistical difference was found in other laboratory indicators, such as total bilirubin (TBIL), alanine transaminase (ALT), aspartic transaminase (AST), γ-glutamyl transferase (γ-GT), albumin, white blood cell (WBC) count, hemoglobin, platelet count, international normalized ratio (INR), and estimated glomerular filtration rate (eGFR) ($P < 0.050$ for all). The frequency of administration of diuretics, anti-ventricular remodeling agents, and β-blocker increased apparently with the progression of cirrhosis ($P \leq 0.001$ for all). The clinical manifestations, laboratory indicators, and CCM medications listed in Table 2 were basically consistent with the clinical practice.

RDW as an independent diagnostic indicator for CCM

The pairwise comparison of the four groups revealed a significant difference for the RDW value that rose constantly with the increasing severity of cirrhosis (12.54 ± 0.85, 13.29 ± 1.19, 14.30 ± 1.96, and 16.25 ± 2.13, $P < 0.001$) (Table 2). All indexes with $P < 0.05$ in Tables 1 and 2 are summarized in Table 3. Multivariate logistics regression analysis was divided into three parts, with the first, second, and third parts corresponding to risk factor analysis of Group II, Group III, and Group IV (Group I as the control group), respectively. Part I reported statistical significance in RDW (odds ratio [OR]: 2.175, 95%CI: 1.549-3.054, $P < 0.001$) between Group II and the control group, with no significant differences in other indicators, which suggests RDW as an independent risk factor in Part I. Part II revealed a marked difference in RDW (OR: 2.447, 95%CI: 1.375-4.354, $P = 0.002$), ALB, ALT, AST, γ-GT, WBC count, hemoglobin, platelet count, INR, NT-proBNP, and eGFR ($P < 0.05$, respectively). Further multivariate logistic regression reported significant differences in RDW (OR: 4.863, 95%CI: 2.493-9.483, $P < 0.001$), ALB, ALT, AST, WBC count, hemoglobin, platelet count, INR, NT-proBNP, and edema ($P < 0.05$, respectively), which were considered as independent risk factors in Part III. Besides, as shown in Table 4, the linear regression analysis showed a positive correlation between RDW and Child-Pugh scores ($r = 0.642$, $P < 0.001$) (Figure 1A) and between RDW and MELD scores ($r = 0.592$, $P < 0.001$) (Figure 1B).

A strong correlation between RDW and different Child-Pugh levels

In Table 2, due to the distribution abnormality in the data of NT-proBNP, the independent sample Kruskal-Wallis test was employed for analysis. The results showed no significant increase in NT-proBNP when the cardiomyopathy cases (Group II) were compared with the non-cardiomyopathy cases (Group I) [76 (61, 98) *vs* 72 (61, 84), $P > 0.05$] but huge differences for both Group III and Group IV ($P < 0.001$ for both). Similarly, the multivariate logistic regression reported no significant difference in NT-proBNP between Group I and Group II (OR: 1.001, 95%CI: 0.997-1.005, $P = 0.518$). The linear regression analysis (Figure 1C) further revealed a positive correlation between RDW and NT-proBNP ($r = 0.715$, $P < 0.001$) (Table 4). Finally, the ROC curve analysis was performed to determine the critical value of continuous variables (RDW and NT-proBNP) for identifying diastolic dysfunction of cardiomyopathy in cirrhosis. The criterion for the selection of optimal cut-off points was comprehensive optimization results of sensitivity and specificity. An obvious difference of P values was found for RDW and NT-proBNP (Figure 2), with a respective AUC of 0.686 (95%CI: 0.624-0.748, $P < 0.001$) and 0.556 (95%CI: 0.490-0.623, $P > 0.050$). The threshold value in ROC curves indicated that LVDD in CCM was more likely to occur if RDW was above 13.05%, with a sensitivity and specificity of 56.0% and 71.4%, respectively (Table 5).

DISCUSSION

As a chronic cardiac dysfunction, CCM is prevalent in 30%-70% of patients with cirrhosis in recent years [32,33] and features a blunted contractile response to stress and altered diastolic relaxation[34]. CCM manifests a complex progression, which depends on the systemic changes resulting from cirrhosis[35].

Table 1 Comparison of demographic and medical records in the study groups, *n* (%)

Variable	Group I (*n* = 105)	Group II (*n* = 175)	Group III (*n* = 139)	Group IV (*n* = 86)	F/χ^2	*P*
Demographics						
Age (yr)	66.20 ± 7.93	67.94 ± 7.41	67.74 ± 7.84	68.00 ± 7.86	1.344	0.259
Male	65 (61.9)	95 (54.3)	82 (59.0)	56 (65.1)	3.304	0.347
Smokers	29 (27.6)	49 (28.0)	51 (36.7)	32 (37.2)	4.697	0.195
Body-mass index (kg/m^2)	23.11 ± 2.37	23.23 ± 3.95	23.75 ± 3.24	24.27 ± 3.52	2.520	0.057
Medical history						
Hypertension	20 (19.0)	32 (18.3)	35 (25.2)	26 (30.2)	6.041	0.110
Hyperlipidemia	20 (19.0)	50 (28.6)	68 (48.9)	40 (46.5)	31.658	< 0.001
Diabetes mellitus	23 (21.9)	35 (20.0)	37 (26.6)	26 (30.2)	4.146	0.246
Atrial fibrillation	8 (7.6)	36 (20.6)	52 (37.4)	34 (39.5)	38.947	< 0.001
Stroke	3 (2.9)	5 (2.9)	4 (2.9)	4 (4.7)	0.908[1]	0.864
Cirrhosis etiology						
Fatty liver	17 (16.2)	22 (12.6)	17 (12.2)	17 (19.8)	3.277	0.351
Alcoholic liver	18 (17.1)	38 (21.7)	32 (23)	26 (30.2)	4.748	0.191
Viral hepatitis	53 (50.5)	100 (57.1)	75 (54)	36 (41.9)	5.695	0.127
Autoimmune liver disease	16 (15.2)	15 (8.6)	14 (10.1)	9 (10.5)	3.157	0.368
Liver cancer	5 (4.8)	8 (4.6)	7 (5)	4 (4.7)	0.151[1]	1.000

[1]Fisher's exact test.

Meanwhile, different criteria may indicate a distinct prevalence. A comparison between the 2005 Montreal criteria and 2019 CCC criteria[3,7] reports a higher prevalence of diastolic dysfunction by the Montreal criteria (64.8% *vs* 7.4%) and a higher proportion of cases with systolic dysfunction by the CCC criteria (16.4% *vs* 53.3%). These cases with systolic dysfunction can only be detected by echocardiography in the presence of stress[36-38]. Unlike systolic dysfunction, diastolic dysfunction appears early and can be detected at the baseline and most patients with cirrhosis display a certain degree of diastolic dysfunction[1,3,37]. Therefore, it is imminent to pinpoint some objective biomarkers before the condition deteriorates into HF.

As a traditional biomarker for the diagnosis and prognostic evaluation of HF, the clinical application of NT-proBNP is quite limited due to multiple factors[39], such as an advanced age and renal dysfunction[40]. Its effectiveness is further compromised in cases of HF with preserved ejection fraction (HFpEF), due to the impact from the clinical characteristics of HFpEF, including obesity, atrial fibrillation, and renal impairment[41]. Moreover, NT-proBNP levels may appear normal in the HFpEF patients, thereby impacting the accuracy of risk assessment[42]. Therefore, it is of great significance to find new biomarkers for the early detection of CCM.

As a hematological parameter, RDW indicates the level of anisocytosis *in vivo*. It can be assessed rapidly and economically. In acute HF patients in the emergency department (ED)[43], advanced HF patients immediately before orthotopic heart transplantation[44], and LVDD in patients with advanced stage of chronic kidney disease (CKD)[45], RDW has always been regarded as an independent predictor. Furthermore, growing evidence has confirmed an inextricable link between RDW and various types of liver-related disorders. Studies have reported the predictive value of RDW for the histological severity of primary biliary cholangitis[15] and its positive correlations with chronic hepatitis B virus infection[16,46-48], non-alcoholic fatty liver disease[49,50], and autoimmune hepatitis[17]. Altogether, these findings lend strong support to our speculation that RDW has unique value in the diagnosis of cirrhosis.

In the current study, the regression analysis showed that RDW was the only significant indicator when Group I was compared with Group II (Table 3, Part I), which highlights that RDW is the only abnormal index in the early stage of CCM. Further linear regression analysis revealed a strong positive correlation between RDW and Child-Pugh (Figure 1A) and MELD scores (Figure 1B), indicating that RDW can serve as an evaluation index for the progression of cirrhosis. As a routine parameter of blood laboratory results, RDW is closely related to HF[11,44,51]. Although the exact underlying mechanism remains controversial, several pathophysiological mechanisms of RDW increase in HF have been proposed, such as inflammation, oxidative stress, adrenergic stimulation, undernutrition, ineffective erythropoiesis, and reduced iron mobilization[51]. Studies have speculated about the link between

Table 2 Comparison of clinical data among groups, *n* (%)

Variable	Group I (*n* = 105)	Group II (*n* = 175)	Group III (*n* = 139)	Group IV (*n* = 86)	F/χ^2	*P*
Clinical feature						
Haematemesis	6 (5.7)	12 (6.9)	16 (11.5)	16 (18.6)	11.588	0.009
Edema	2 (1.9)	12 (6.9)	20 (14.4)	33 (38.4)	65.284	< 0.001
SBP	1 (1.0)	4 (2.3)	11 (7.9)	13 (15.1)	23.486	< 0.001
Hyponatremia	3 (2.9)	21 (12.0)	31 (22.3)	31 (36.0)	42.806	< 0.001
Hepatic encephalopathy	5 (4.8)	10 (5.7)	14 (10.1)	19 (22.1)	21.565	< 0.001
Refractory ascites	2 (1.9)	4 (2.3)	15 (10.8)	21 (24.4)	44.367	< 0.001
Jaundice	12 (11.4)	13 (7.4)	15 (10.8)	20 (23.3)	14.120	0.003
MELD score	8.74 ± 1.54	8.64 ± 1.57	17.04 ± 1.96[a,b]	25.19 ± 2.15[a,b,c]	2081.425	< 0.001
Laboratory index						
RDW (%)	12.54 ± 0.85	13.29 ± 1.19[a]	14.3 ± 1.96[a,b]	16.25 ± 2.13[a,b,c]	101.958	< 0.001
TBIL (μmol/L)	12.3 (10.9, 13.45)	11.5 (9.8, 14.5)	12.8 (10.7, 17.5)[b]	16.3 (13.18,24.95)[a,b,c]	57.344	< 0.001
ALB (g/L)	45.91 ± 3.83	44.92 ± 5.19[a]	37.85 ± 4.71[a,b]	33.50 ± 5.65[a,b,c]	159.072	< 0.001
ALT (IU/L)	24 (18, 32)	27 (21, 34)[a]	32 (23, 37)[a,b]	35 (26, 75)[a,b,c]	40.591	< 0.001
AST (IU/L)	26 (19.5, 33)	28 (21, 35)	30 (22, 36)[a]	34 (25, 62)[a,b]	25.257	< 0.001
γ-GT (IU/L)	32 (20, 44)	30 (22, 41)	35 (26, 43)	37 (26, 47)[a,b]	14.96	0.002
ALP (IU/L)	65 (52, 84)	74 (53, 88)	69 (54, 87)	69 (58, 84)	3.535	0.316
WBC count (× 10^9/L)	5.64 ± 1.51	5.36 ± 1.40	5.76 ± 1.16[b]	6.10 ± 1.55[a,b]	5.695	0.001
Hemoglobin (g/L)	122.86 ± 15.53	121.10 ± 15.30	107.92 ± 16.33[a,b]	99.28 ± 18.15[a,b,c]	52.186	< 0.001
Platelet count (× 10^9/L)	190.15 ± 62.47	174.98 ± 41.71[a]	101.09 ± 27.46[a,b]	90.38 ± 32.33[a,b]	165.715	< 0.001
INR	0.91 (0.59, 1.27)	1.21 (0.61, 1.80)[a]	1.58 (0.86, 2.41)[a,b]	1.37 (1.17, 2.22)[a,b]	57.797	< 0.001
NT-proBNP (pg/mL)	72 (61, 84)	76 (61, 98)	354 (127, 653)[a,b]	647 (364, 1430)[a,b,c]	242.974	< 0.001
eGFR (ml/min/1.73 m^2)	62.35 ± 11.09	60.87 ± 13.52	59.07 ± 15.94	43.31 ± 11.39[a,b,c]	40.691	< 0.001
Drug information						
Diuretic	3 (2.9)	7 (4)	20 (14.4)	32 (37.2)	69.996	< 0.001
Spirolactone	3 (2.9)	7 (4)	17 (12.2)	26 (30.2)	50.491	< 0.001
ACEI/ARB	7 (6.7)	23 (13.1)	22 (15.8)	23 (26.7)	15.690	0.001
β-blocker	6 (5.7)	13 (7.4)	23 (16.5)	22 (25.6)	23.776	< 0.001

[a]*P* < 0.05 when compared with Group I.
[b]*P* < 0.05 when compared with Group II.
[c]*P* < 0.05 when compared with Group III.
ACEI: Angiotensin converting enzyme inhibitor; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine transaminase; ARB: Angiotensin receptor blocker; AST: Aspartic transaminase; eGFR: Estimated glomerular filtration rate; INR: International normalized ratio; NT-proBNP: N-terminal pro-brain natriuretic peptide; RDW: Red blood cell distribution width; SBP: Spontaneous bacterial peritonitis; TBIL: Total bilirubin; WBC: White blood cell; γ-GT: γ-glutamyl transferase.

impaired hematopoiesis and cardiac dysfunction, given the concurrent presence of increased heterogeneity of erythrocyte volume in HF patients and the contributing role of anisocytosis in the HF deterioration[52]. Meanwhile, studies have also suggested NT-proBNP as an important index for the diagnosis and evaluation of HF[39]. In the current study, the comparison of NT-proBNP between groups II, III, and IV in Table 2 showed that HFpEF deteriorated with the worsening severity of cirrhosis, which was similar to the increasing tendency of RDW in the aggravating Child-Pugh levels. Nevertheless, unlike RDW, NT-proBNP was not a percipient of cardiomyopathy in patients with Child-Pugh Class A cirrhosis, which was verified by the multiple logistic regression analysis in the first part of Table 3. In order to further expound the relationship between the two indicators, the linear regression analysis was performed between RDW and NT-proBNP (Figure 1C, Table 4), which showed that they

Table 3 Multiple logistics regression analysis for each group and the control group

Part	Variable	B	SE	Wald	*P*	OR (95%CI)
Part I	RDW (%)	0.777	0.173	20.141	< 0.001	2.175 (1.549, 3.054)
	TBIL (μmol/L)	-0.086	0.051	2.810	0.094	0.918 (0.83, 1.015)
	ALB (g/L)	-0.060	0.035	2.973	0.085	0.942 (0.879, 1.008)
	ALT (IU/L)	0.122	0.067	3.350	0.067	1.129 (0.991, 1.287)
	AST (IU/L)	-0.108	0.060	3.178	0.075	0.898 (0.798, 1.011)
	γ-GT (IU/L)	0.004	0.011	0.117	0.733	1.004 (0.982, 1.026)
	WBC count (× 10^9/L)	0.026	0.107	0.059	0.808	1.026 (0.832, 1.265)
	Hemoglobin (g/L)	0.012	0.010	1.294	0.255	1.012 (0.992, 1.032)
	Platelet count (× 10^9/L)	-0.006	0.003	3.638	0.056	0.994 (0.988, 1.000)
	INR	0.418	0.290	2.074	0.150	1.519 (0.86, 2.683)
	NT-proBNP (pg/mL)	0.001	0.002	0.417	0.518	1.001 (0.997, 1.005)
	eGFR (mL/min/1.73 m^2)	-0.009	0.012	0.598	0.439	0.991 (0.967, 1.015)
	Hyperlipidemia	-0.074	0.346	0.046	0.831	0.929 (0.471, 1.830)
	Atrial fibrillation	-0.402	0.602	0.448	0.503	0.669 (0.206, 2.174)
	Haematemesis	-0.730	0.711	1.054	0.305	0.482 (0.12, 1.942)
	Edema	0.105	1.196	0.008	0.930	1.111 (0.107, 11.580)
	SBP	1.623	2.242	0.524	0.469	5.07 (0.063, 410.934)
	Hyponatremia	-1.682	0.934	3.243	0.072	0.186 (0.030, 1.16)
	Hepatic encephalopathy	-0.161	0.804	0.040	0.842	0.852 (0.176, 4.119)
	Jaundice	-1.044	1.264	0.683	0.409	0.352 (0.030, 4.188)
	Refractory ascites	0.256	1.498	0.029	0.864	1.292 (0.069, 24.324)
Part II	RDW (%)	0.895	0.294	9.265	0.002	2.447 (1.375, 4.354)
	TBIL (μmol/L)	-0.028	0.083	0.116	0.733	0.972 (0.825, 1.145)
	ALB (g/L)	-0.723	0.140	26.552	< 0.001	0.485 (0.369, 0.639)
	ALT (IU/L)	0.337	0.087	15.167	< 0.001	1.401 (1.182, 1.660)
	AST (IU/L)	-0.390	0.087	19.883	< 0.001	0.677 (0.571, 0.804)
	γ-GT (IU/L)	0.037	0.018	4.313	0.038	1.037 (1.002, 1.074)
	WBC count (× 10^9/L)	0.592	0.253	5.464	0.019	1.807 (1.100, 2.969)
	Hemoglobin (g/L)	-0.057	0.026	4.929	0.026	0.944 (0.898, 0.993)
	Platelet count (× 10^9/L)	-0.055	0.010	32.104	< 0.001	0.947 (0.929, 0.965)
	INR	1.894	0.563	11.321	0.001	6.643 (2.205, 20.018)
	NT-proBNP (pg/mL)	0.008	0.003	8.203	0.004	1.008 (1.003, 1.014)
	eGFR (mL/min/1.73 m^2)	0.062	0.027	5.365	0.021	1.064 (1.010, 1.121)
	Hyperlipidemia	-1.173	0.658	3.174	0.075	0.309 (0.085, 1.125)
	Atrial fibrillation	0.365	1.006	0.132	0.717	1.441 (0.201, 10.345)
	Haematemesis	2.282	1.499	2.318	0.128	9.792 (0.519, 184.678)
	Edema	3.004	1.778	2.855	0.091	20.159 (0.618, 657.095)
	SBP	4.715	4.750	0.985	0.321	111.572 (0.010, 1231574.165)
	Hyponatremia	-0.424	1.259	0.113	0.736	0.655 (0.055, 7.724)
	Hepatic encephalopathy	-1.850	1.290	2.057	0.151	0.157 (0.013, 1.970)

	Jaundice	1.862	2.392	0.606	0.436	6.437 (0.059, 699.588)
	Refractory ascites	-1.431	3.819	0.140	0.708	0.239 (0, 425.671)
Part III	RDW (%)	1.582	0.341	21.541	< 0.001	4.863 (2.493, 9.483)
	TBIL (μmol/L)	-0.080	0.090	0.787	0.375	0.923 (0.774, 1.101)
	ALB (g/L)	-0.910	0.150	37.020	< 0.001	0.402 (0.300, 0.539)
	ALT (IU/L)	0.350	0.087	16.273	< 0.001	1.419 (1.197, 1.682)
	AST (IU/L)	-0.410	0.088	21.725	< 0.001	0.664 (0.559, 0.789)
	γ-GT (IU/L)	0.032	0.018	3.226	0.072	1.033 (0.997, 1.070)
	WBC count (× 10^9/L)	0.763	0.292	6.798	0.009	2.144 (1.208, 3.803)
	Hemoglobin (g/L)	-0.070	0.029	5.697	0.017	0.932 (0.880, 0.988)
	Platelet count (× 10^9/L)	-0.077	0.013	35.070	< 0.001	0.926 (0.902, 0.950)
	INR	1.828	0.621	8.661	0.003	6.224 (1.842, 21.031)
	NT-proBNP (pg/mL)	0.008	0.003	7.937	0.005	1.008 (1.002, 1.014)
	eGFR (mL/min/1.73 m^2)	-0.005	0.033	0.018	0.892	0.995 (0.932, 1.063)
	Hyperlipidemia	-0.404	0.764	0.280	0.597	0.667 (0.149, 2.986)
	Atrial fibrillation	0.417	1.132	0.135	0.713	1.517 (0.165, 13.954)
	Haematemesis	3.164	1.693	3.493	0.062	23.67 (0.857, 653.530)
	Edema	3.826	1.882	4.132	0.042	45.891 (1.147, 1836.043)
	SBP	5.570	4.815	1.339	0.247	262.503 (0.021, 3291299.144)
	Hyponatremia	-0.376	1.364	0.076	0.783	0.687 (0.047, 9.940)
	Hepatic encephalopathy	-1.949	1.468	1.763	0.184	0.142 (0.008, 2.530)
	Jaundice	0.527	2.529	0.043	0.835	1.694 (0.012, 240.821)
	Refractory ascites	-0.538	3.896	0.019	0.890	0.584 (0, 1210.714)

The multiple logistics regression reference group was Group I. Child-Pugh group assignment: Group I = 0, Group II = 1, Group III = 2, Group IV = 3. Every count indicator was set to 0 if unavailable and 1 if available. ALB: Albumin; ALT: Alanine transaminase; AST: Aspartic transaminase; eGFR: Estimated glomerular filtration rate; INR: International normalized ratio; NT-proBNP: N-Terminal pro-brain natriuretic peptide; RDW: Red blood cell distribution width; SBP: Spontaneous bacterial peritonitis; TBIL: Total bilirubin; WBC: White blood cell; γ-GT: γ-glutamyl transferase.

Table 4 Pearson correlation analysis between red blood cell distribution width and each index

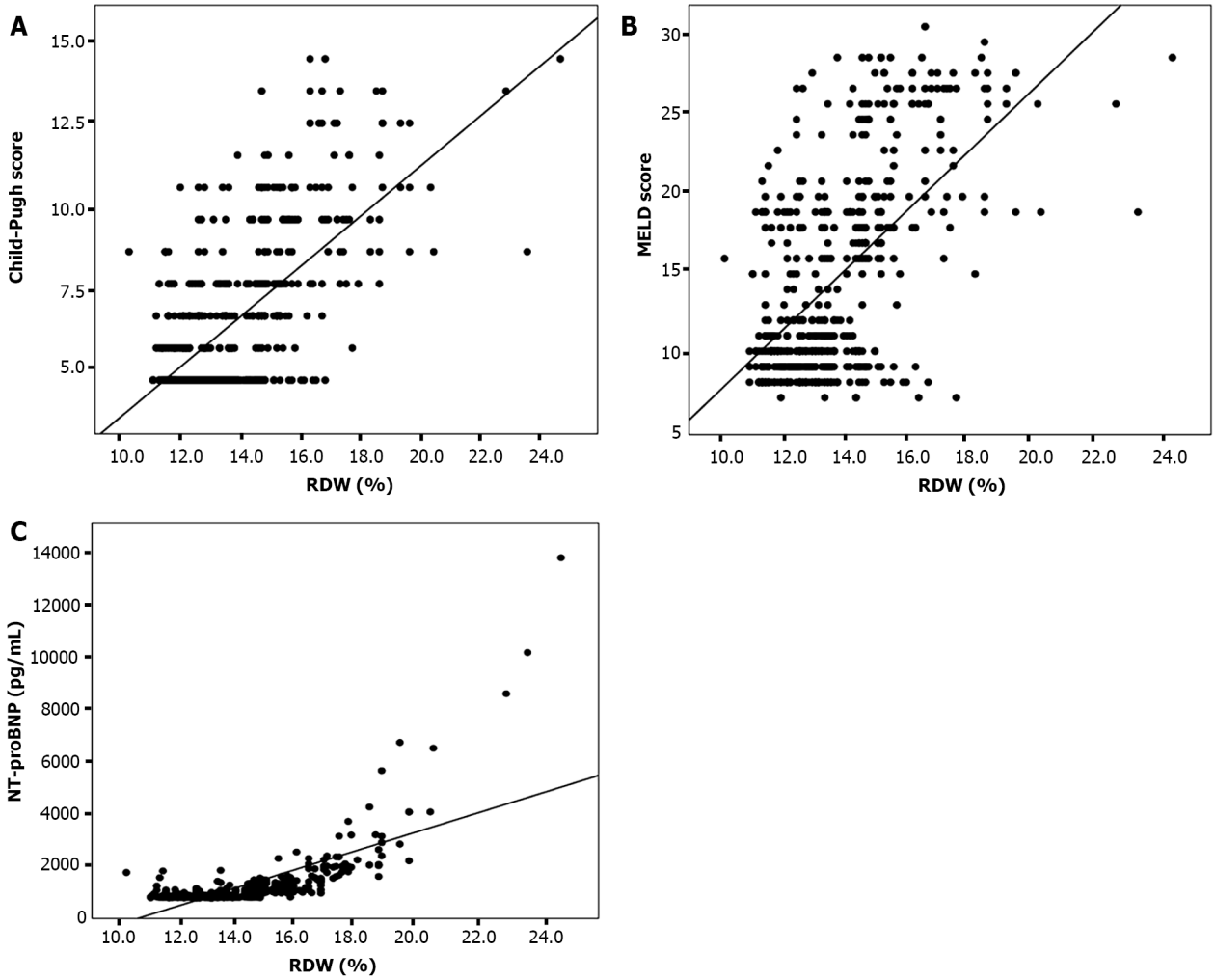
		Child-Pugh score	MELD score	NT-proBNP
RDW	*r* value	0.642	0.592	0.715
	P value	< 0.001	< 0.001	< 0.001

MELD score: Model for End-Stage Liver Disease score; NT-proBNP: N-terminal pro-brain natriuretic peptide; RDW: Red blood cell distribution width.

Table 5 Receiver operating characteristic curve analysis of key indexes among groups

Variable	AUC (95%CI)	SE	*P*	Critical value	Sensitivity	Specificity	Youden index
RDW	0.686 (0.624, 0.748)	0.032	< 0.001	13.05	56.0%	71.4%	0.274
NTproBNP	0.556 (0.490, 0.623)	0.034	0.114	95.50	28.0%	89.5%	0.175

AUC: Area under the curve; CI: Confidence interval; NT-proBNP: N-terminal pro-brain natriuretic peptide; RDW: Red blood cell distribution width.

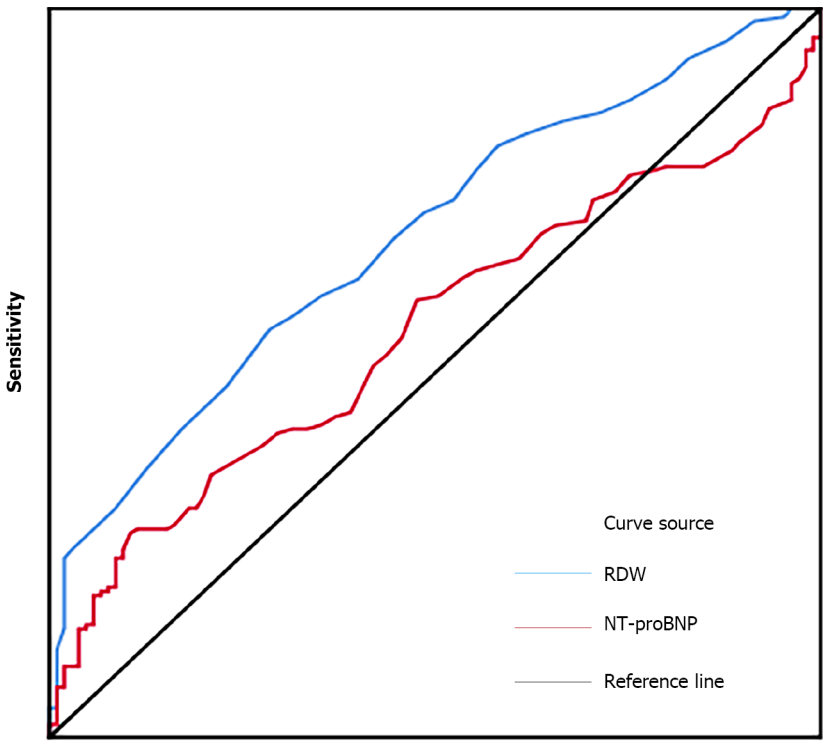


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Figure 1 Correlation. A: Correlation between red blood cell distribution width (RDW) and Child-Pugh scores; B: Correlation between RDW and Model for End-Stage Liver Disease scores; C: Correlation between RDW and N-terminal pro-brain natriuretic peptide. RDW: Red blood cell distribution width; NT-proBNP: N-terminal pro-brain natriuretic peptide.

were positively correlated with different slopes. The ROC curve analysis on this basis (Figure 2, Table 5) revealed that for mild cases of cirrhosis, the possibility of cardiomyopathy increased (sensitivity 56.0%, specificity 71.4%) when RDW was greater than 13.05%, while the change of NT-proBNP was not significant (P = 0.114). Taken together, these findings suggest that compared with NT-proBNP, RDW can serve as a more sensitive indicator for the early stage of CCM.

The above finding may be accounted for with the following explanations. First, cirrhosis is a chronic inflammatory disease characterized by gradual necrosis of hepatocytes, which causes a systemic inflammatory response, a slow but irreversible decline in liver function, and the development of portal hypertension[53]. On the one hand, the pathological function of the liver can increase anisocytosis and reduce erythrocyte deformability and oxygen-carrying capacity, which may further result in reduced peripheral and myocardial tissue oxygenation, contributing to HF. On the other hand, inflammatory cytokines that are closely related to cirrhosis also play an important role in the pathogenesis of HF[54], which may affect the maturation and speed of erythrocytes. Moreover, the entry of younger and larger reticulocytes into the peripheral circulation may be potential reasons for increasing RDW[50]. Still, inflammation may impair the bone marrow function, resulting in the release of premature senescent erythrocytes into the circulation and RDW increase[28,51]. Malnutrition, a common complication of liver disease, has been shown to be involved in RDW increase in HF[51]. Finally, portal hypertension due to cirrhosis can cause hemodynamic abnormalities and hypersplenism, thus accelerating the changes in erythrocyte morphology, which is inextricably linked to LVDD[4]. However, the exact mechanism awaits further exploration.



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Figure 2 Sensitivity and specificity of red blood cell distribution width and N-terminal pro-brain natriuretic peptide assesserd by receiver operating characteristic curve analysis. RDW: Red blood cell distribution width; NT-proBNP: N-terminal pro-brain natriuretic peptide.

CONCLUSION

Compared with NT-proBNP, RDW displays a higher sensitivity in the prediction of LVDD in CCM, especially in forepart hepetocirrhosis. A significant risk of CCM may be indicated if the quantitative RDW value of more than 13.05% is reported.

ARTICLE HIGHLIGHTS

Research background

Cirrhotic cardiomyopathy (CCM) was originally derived from studies of perioperative heart failure (HF) in liver transplant patients. In recent years, more and more researchers have found that not only patients undergoing liver transplantation, but also many patients diagnosed with cirrhosis will have cardiac insufficiency without other organic heart disease. CCM was often found in advanced cirrhosis.

Research motivation

At present, exact diagnostic criteria of echocardiography have been established for CCM. However, in most Chinese hospitals, due to high cost, echocardiography is not a good screening method for cases without clinical manifestations of HF. We are trying to find a proper method to predict CCM in order to achieve early detection and treatment.

Research objectives

To explore suitable biomarkers for early CCM prediction.

Research methods

We adopted the methods of data analysis. Under the premise of clear diagnostic criteria for CCM, risk factors were screened by multivariate regression analysis, and red blood cell distribution width (RDW), Child-Pugh classification, and N-terminal pro-brain natriuretic peptide (NT-proBNP) were analyzed by linear regression, and finally ROC curve analysis was performed to determine the critical value.

Research results

The possibility of cardiomyopathy increased (sensitivity 56.0%, specificity 71.4%) when RDW was greater than 13.05%, while the change of NT-proBNP was not significant (P = 0.114). Taken together, these findings suggest that compared with NT-proBNP, RDW can serve as a more sensitive indicator for the early stage of CCM.

Research conclusions

RDW can serve as an effective and accessible clinical indicator for the prediction of diastolic dysfunction in CCM, in which a numerical value of more than 13.05% may indicate an increasing CCM risk.

Research perspectives

First, large-scale and multi-center studies are needed to reduce the deviation error. Second, continuous hemodynamic monitoring is necessary to further analyze the hemodynamic changes in early cirrhosis.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Reduction of portosystemic gradient during transjugular intrahepatic portosystemic shunt achieves good outcome and reduces complications

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Abstract

BACKGROUND

Transjugular intrahepatic portosystemic shunt (TIPS) is placed important role in the therapy of complications of portal hypertension, there is still no suitable criterion for a reduction in portosystemic gradient (PSG), which can both reduce PSG and maximize clinical results and minimize hepatic encephalopathy (HE).

AIM

To compare the clinical outcomes and incidence of HE after one-third PSG reduction during TIPS in patients with variceal bleeding and refractory ascites.

METHODS

A total of 1280 patients with portal-hypertension-related complications of refractory ascites or variceal bleeding who underwent TIPS from January 2016 to January 2019 were analyzed retrospectively. Patients were divided into group A (variceal hemorrhage and PSG reduced by one third, n = 479); group B (variceal hemorrhage and PSG reduced to < 12 mmHg, n = 412); group C (refractory ascites and PSG reduced by one third, n = 217); and group D (refractory ascites and PSG reduced to < 12 mmHg of PSG, plus medication, n = 172). The clinical outcomes

were analyzed.

RESULTS

By the endpoint of follow-up, recurrent bleeding was no different between groups A and B (χ^2 = 7.062, P = 0.374), but recurrent ascites did differ significantly between groups C and D (χ^2 = 14.493, P = 0.006). The probability of total hepatic impairment within 3 years was significantly different between groups A and B (χ^2 = 11.352, P = 0.005) and groups C and D (χ^2 = 13.758, P = 0.002). The total incidence of HE differed significantly between groups A and B (χ^2 = 7.932, P = 0.016), groups C and D (χ^2 = 13.637, P = 0.007). There were no differences of survival rate between groups A and B (χ^2 = 3.376, P = 0.369, log-rank test), but did differ significantly between groups C and D (χ^2 = 13.582, P = 0.014, log-rank test).

CONCLUSION

The PSG reduction by one third may reduce the risk of HE, hepatic function damage and achieve good clinical results.

Key Words: Portal hypertension; Transjugular intrahepatic portosystemic shunt; Portosystemic gradient; Liver cirrhosis; Variceal bleeding; Refractory ascites

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Core Tip: Patients with cirrhosis who underwent transjugular intrahepatic portosystemic shunt for recurrent variceal bleeding and refractory ascites were evaluated. Reduction in portosystemic gradient (PSG) should be based on the original basal pressure and reduction by one third may reduce the risk of hepatic encephalopathy, hepatic function damage and achieve similar clinical results as for the refractory ascites patients. Appropriate reduction of PSG directly influences the patient prognosis.

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is placed important role in the therapy of complications of portal hypertension[1], It has been progressively recognized as an effective therapeutic option in a growing number of clinical situations[2,3]. Measurement of portosystemic pressure gradient (PSG) is important during the TIPS procedure. Reduction of PSG can achieve good clinical results, but when PSG is too low, TIPS can have many complications, of which, hepatic encephalopathy (HE) and liver function damage are the most frequent[4]. Post-TIPS HE could depend mainly on portocaval pressure gradient and volume of blood shunted through the liver[5].

Several guidelines[6,7] recommend that the PSG should be reduced to 12 mmHg after TIPS creation to achieve a better clinical outcome. In that situation, however, the incidence of HE is higher than in clinical practice. This has prompted many centers to anecdotally adopt the technique of dilation of stent grafts using balloons with a nominal diameter of ≤ 8 mm at TIPS positioning. Recently, a new controlled expansion stent has been introduced in clinical practice (Viatorr Controlled Expansion Endoprosthesis; Gore and Associates, Flagstaff, AZ, United States), which allows lasting diameter control within a range of 8–10 mm during implantation to reach a targeted portal pressure gradient[8].

However, there is still no suitable criterion for a reduction in PSG, which can both reduce PSG and maximize clinical results and minimize HE, and few data are available to calculate an appropriate PSG value[9]. Here, we report our multicenter retrospective study to compare the occurrence of HE and clinical results of one-third reduction of PSG with PSG reduced to < 12 mmHg in patients who required TIPS placement.

MATERIALS AND METHODS

Patient information

This was a multicenter retrospective study. The Ethics Committee approved the study protocol and all procedures were conducted according to the guidelines approved by the Committee. Between January 2016 and January 2019, 1280 patients were referred on an intention-to-treat basis and underwent a TIPS procedure. Indications for stent graft shunt were variceal hemorrhage or refractory ascites. The outcomes of HE, recurrent variceal bleeding and ascites, and mortality were compared between the groups. The patients' medical records and images were reviewed to gather information regarding underlying etiology, clinical presentation, age, sex, and severity of cirrhosis (Table 1).

Study design

This was a multicenter retrospective study that compared the rate of HE and clinical outcomes after TIPS with PSG reduced by one third with PSG reduced to < 12 mmHg in patients who required TIPS placement for portal-hypertension-related complications of ascites or variceal bleeding. The patients were divided into four groups[10]: Group A (variceal hemorrhage and PSG reduced by one third, n = 479); group B (variceal hemorrhage and PSG reduced to < 12 mmHg, n = 412); group C (refractory ascites and PSG reduced by one third, n = 217); and group D (refractory ascites and PSG reduced to < 12 mmHg, plus medication, n = 172). The clinical outcomes were analyzed.

The inclusion criteria were: Recurrent variceal bleeding after a session of variceal sclerotherapy, and refractory ascites that required TIPS placement with portal-hypertension-related complications. Only *de novo* TIPS procedures using Viatorr stent grafts (Gore & Associates) were included. We excluded: TIPS procedures performed with bare stents, TIPS with bare stents followed by revision with Viatorr stent grafts, and TIPS performed with other types of stent grafts; variceal bleeding as an emergency indication; portal vein thrombosis; history of HE; severe right-sided heart failure; severe liver failure (bilirubin > 4 mg/dL), polycystic liver disease, and dilated biliary ducts; age > 75 years; Child–Pugh score > 11; Model of End-Stage Liver Disease (MELD) score > 18; hepatic carcinoma; sepsis spontaneous bacterial peritonitis; and liver transplantation.

TIPS procedure

TIPS was performed under standard local anesthesia as described previously[11]. The entire length of the intrahepatic tract was covered by the stent graft. Hepatic venous pressure gradient and portal venous pressure were measured during the procedure, and the shunts were dilated to an appropriate diameter to reach a target PSG of < 12 mmHg or reduced by one third. To reduce PSG by one third of basal value, the stent was not fully expanded, the diameter was retained, and the pressure was measured several times until it was reduced by one third. Obvious gastroesophageal collateral vessels observed during the TIPS procedure were embolized with coils (Cook Inc., Bloomington, IL, United States; or Interlock Coil, Boston Scientific Corporation, Natikeshi, MA, United States). Subsequent direct portography was performed to evaluate whether the portal venous system was completely patent.

After the TIPS procedure, intravenous Dalteparin Sodium Injection (5000 U/d; VetterPharma-Fertigung, Germany) was administered for 3 d. No patients had portal vein thrombosis, and oral warfarin was not given.

Follow-up

After TIPS deployment, baseline duplex sonography was performed. Shunt velocities were compared with this baseline result during follow-up. Patients were placed into a routine follow-up protocol identical for each group. They were seen as outpatients 1 mo after the procedure and then at 3 and 6 mo and 1 and 3 years, or whenever needed. Each consultation included a clinical examination, blood chemistry, upper abdominal ultrasonography, and assessment of HE.

TIPS angiography was performed in patients with recurrent symptoms or suspected shunt dysfunction. TIPS revision was performed when hemodynamically significant shunt stenosis (> 50%) was present with recurrent variceal bleeding, or recurrent or gradually worsening ascites. HE was defined according to the practice guidelines of the European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD)[12,13]. Patients lost to follow-up were censored at the time of the last known imaging of the shunt (duplex ultrasonography or shunt venography).

Statistical analysis

Data measurements results of the four groups were normally distributed, and expressed as mean ±SD, and their differences were determined using t test. Categorical variables were expressed as frequencies and compared using χ^2 test, and their differences among the four groups were determined by one-way ANOVA. A P value of less than 0.05 was considered significant. The statistical analyses were performed with SPSS version 22.0 (SPSS, Armonk, NY, United States).

Table 1 Baseline characteristics

Characteristics	Group A	Group B	*P* value	Group C	Group D	*P* value
Gender, M/F	256/223	237/175	0.369	126/91	93/79	0.319
Age (mean ± SD) (yr)	54.616 ± 17.27	56.39 ± 12.19	0.319	56.24 ± 13.67	58.27 ± 13.25	0.246
Child–Pugh A/B/C	39/342/98	32/318/62	0.187	0/60/157	0/41/131	0.215
MELD score (mean ± SD)	8.42 ± 1.37	9.29 ± 2.16	0.576	13.26 ± 4.56	14.39 ± 5.38	0.472
Viral hepatitis	324	276	0.528	136	107	0.632
Chronic ethanol consumption	102	87	0.317	55	38	0.258
Cryptogenic hepatitis	53	49	0.492	26	27	0.146
Variceal hemorrhage	479	412	0.721	0	0	0
Refractory ascites	0	0	0	217	172	0.562
Laboratory tests						
Alanine transaminase (U/L)	48.36 ± 4.21	53.19 ± 3.27	0.462	62.13 ± 6.48	57.49 ± 7.29	0.368
Aspartate Transaminase (U/L)	54.17 ± 9.25	58.27 ± 12.37	0.361	67.43 ± 15.7	64.28 ± 17.24	0.357
Alkaline phosphatase (U/L)	145.36 ± 23.45	167.18 ± 27.36	0.382	89.67 ± 13.24	92.36 ± 16.58	0.413
γ-Glutamyl transpeptidase (U/L)	278.54 ± 37.47	259.74 ± 46.37	0.463	364.27 ± 58.74	382.17 ± 47.26	0.482
Total bilirubin (mmol/L)	29.45 ± 3.17	32.46 ± 4.28	0.147	37.18 ± 7.69	35.24 ± 8.54	0.367
Albumin (g/L)	31.28 ± 1.47	32.07 ± 1.25	0.106	28.07 ± 1.29	29.36 ± 1.48	0.294
Prothrombin time (s)	14.02 ± 1.35	15.04 ± 1.19	0.236	18.12 ± 2.39	19.23 ± 2.41	0.241
Clinical presentations						
Abdominal distention	89	93	0.261	217	172	0.562
Abdominal pain	48	52	0.273	134	97	0.183
Weakness	364	314	0.148	189	154	0.136
Poor appetite	373	362	0.302	196	153	0.324
Jaundice	28	24	0.532	21	16	0.214
Splenomegaly	264	249	0.357	205	168	0.436
Lower limbs edema	47	62	0.159	189	157	0.327
Ascites paracentesis	0	0	0	217	172	0.562
Endoscopic therapy	453	407	0.372	0	0	0

No difference ($P > 0.05$) could be seen in terms of age, sex, Child–Pugh score, MELD score, laboratory tests, and clinical presentations. MELD: Model of End-Stage Liver Disease; SD: Standard deviation; M: Male; F: Female.

RESULTS

All TIPS procedures showed similar efficacy in all four groups by reducing the PSG before and after TIPS. PSG was reduced after TIPS placement from 24.58 ± 2.41 to 15.72 ± 1.04 mmHg in group A (P = 0.012), 25.37 ± 2.54 to 11.27 ± 2.04 mmHg in group B (P = 0.004), 25.12 ± 3.16 to 16.15 ± 1.37 mmHg in group C (P = 0.016), and 24.48 ± 3.24 to 10.28 ± 1.18 mmHg in group D (P = 0.003) (Table 2). It showed that there were significant differences between groups A and B (P = 0.017) and groups C and D (P = 0.026).

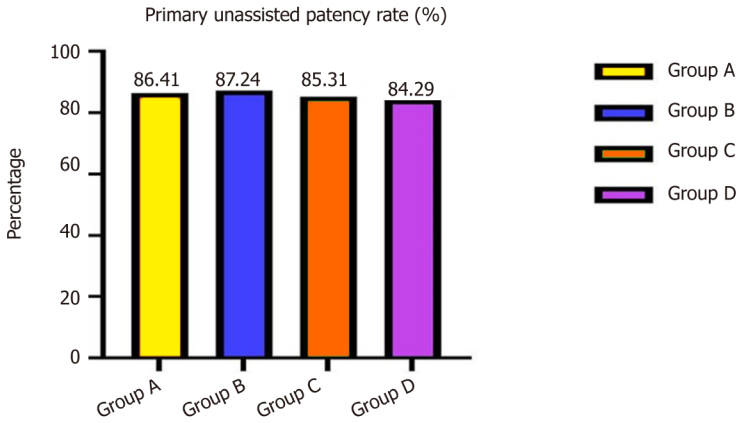
No patient died within 30 d after TIPS, with an early survival of 100%. None of the patients in groups A and B had recurrent bleeding within the first week. The symptoms of ascites in 198 (91.24%) patients in group C and 161 (93.60%) in group D disappeared or were relieved without paracentesis, with no significant differences (P = 0.327).

During 3-years' follow-up, the total primary unassisted patency rates in groups A and B were 86.41% *vs* 87.24% (χ^2 = 4.486, P = 0.257), and in groups C and D were 85.31% *vs* 84.29% (χ^2 = 4.529, P = 0.248), with no significant differences (Figure 1).

Table 2 Portosystemic gradient changes in the two groups

Groups	PSG (mmHg)		*t* value	*P* value
	Before	After		
Group A	24.58 ± 2.41	15.72 ± 1.04	11.48	0.012
Group B	25.37 ± 2.54	11.27 ± 2.04	14.25	0.004
	(*t* value) 0.649	6.382		
	(*P* value) 0.483	0.026		
Group C	25.12 ± 3.16	16.15 ± 1.37	12.43	0.016
Group D	24.48 ± 3.24	10.28 ± 1.18	15.47	0.003
	(*t* value) 0.367	5.734		
	(*P* value) 0.534	0.017		

There are differences before and after transjugular intrahepatic portosystemic shunt in the four groups: group A compared with B, and group C compared with D. PSG: Portosystemic gradient.



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Figure 1 Total primary unassisted patency rates of four groups. The total primary unassisted patency rates were not significantly different.

Forty-three (8.97%) patients in group A, 96 (23.30%) in group B, 27 (12.44%) in group C and 63 (36.62%) in group D developed hepatic function compromise after TIPS placement. The probability of total hepatic impairment within 3 years differed significantly between groups A and B (χ^2 = 11.352, *P* = 0.005) and groups C and D (χ^2 = 13.758, *P* = 0.002) (Figure 2). Mean aspartate transaminase, alanine transaminase, and total bilirubin concentrations were elevated, albumin levels decreased, and prothrombin time was prolonged compared with pre-TIPS.

At the end of follow-up, 56 (11.69%) patients in group A and 47 (11.40%) in group B had recurrent variceal bleeding, which was not a significant difference (χ^2 = 7.062, *P* = 0.374) (Figure 3).

Eighty-Nine (41.01%) patients in group C, 126 (73.25%) in group D with recurrent ascites, which was a significant difference (χ^2 = 14.493, *P* = 0.006) (Figure 4).

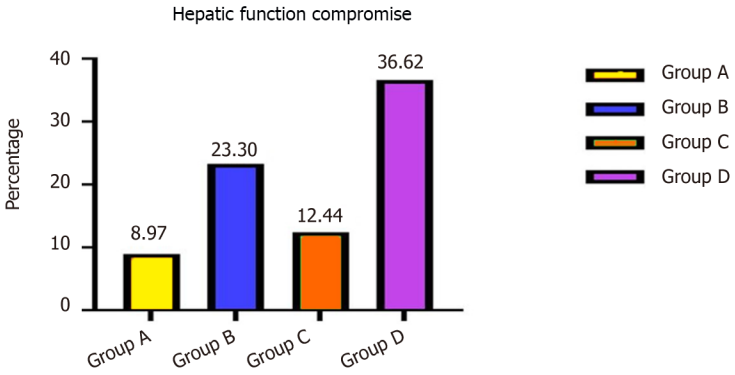
Of these, 27 (5.63%) patients in group A, 21 (5.09%) in group B, 13 (5.99%) in group C and nine (5.23%) in group D were caused by stent dysfunction, and after stent revision, the symptoms disappeared, and there was no significant difference between the groups (χ^2 = 834, *P* = 0.358; χ^2 = 4.574, *P* = 0.375).

The bleeding in patients in groups A and B that was not caused by stent dysfunction was relieved after medical treatment. However, 76 (35.02%) patients in group C and 117 (68.02%) in group D was not caused by stent graft dysfunction but rather hepatic dysfunction and hypoalbuminemia, which differed significantly between the two groups (χ^2 = 13.356, *P* = 0.006). After medication and albumin supplementation, the symptoms recurred many times (Table 3).

Symptoms of variceal bleeding in groups A and B disappeared within 1 wk, and symptoms of ascites in groups C and D disappeared or were relieved within 1 wk without paracentesis, and total primary unassisted patency rates were not significantly different. The probability of total hepatic impairment and recurrent symptoms was significantly different between the groups.

Table 3 Outcomes of symptoms in the four groups

Symptoms	Group A	Group B	χ^2	*P* value	Group C	Group D	χ^2	*P* value
Ascites within 1 wk	/	/		/	198 (198/217, 91.24%)	161 (161/172, 93.60%)		0.327
Hemorrhage within 1 wk	0	0		0	/	/		/
Primary unassisted patency rate	0.8641	0.8724	4.486	0.257	0.8531	0.8429	4.529	0.248
Hepatic function compromise	43 (43/479, 8.97%)	96 (96/412, 23.30%)	11.352	0.005	27 (27/217, 12.44%)	63 (63/172, 36.62%)	13.758	0.002
Recurrence of hemorrhage	56 (56/479, 11.69%)	47 (47/412, 11.40%)	7.062	0.374	/	/	/	/
Stent dysfunction	27 (27/479, 5.63%)	21 (21/412, 5.09%)	6.834	0.358	/	/	/	/
Non-stent dysfunction	29 (29/479, 6.05%)	26 (26/412, 6.31%)	6.486	0.362	/	/	/	/
Recurrence of ascites	/	/	/	/	89 (89/217, 41.01%)	126 (126/172,73.25%)	14.493	0.006
Stent dysfunction	/	/	/	/	13 (13/217, 5.99%)	9 (9/172, 5.23%)	4.574	0.375
Non-stent dysfunction	/	/	/	/	76 (76/217, 35.02%)	117 (117/172, 68.02%)	13.356	0.006



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Figure 2 Hepatic function compromise after transjugular intrahepatic portosystemic shunt. The probability of total hepatic impairment differed significantly between groups A and B, and C and D.

During the 3-year follow-up period, 46 (9.60%) patients in group A, 67 (16.26%) in group B, 47 (21.65%) in group C and 69 (40.11%) in group D developed HE, and the incidence of HE in group A compared with group B, and group C compared with group D differed significantly (χ^2 = 7.932, *P* = 0.016; χ^2 = 13.637, *P* = 0.007, respectively). There were significant differences in the occurrence of HE between groups A and B at 1 mo (χ^2 = 6.463, *P* = 0.027), 3 mo (χ^2 = 5.0368, *P* = 0.023), 6 mo (χ^2 = 6.473, *P* = 0.017), 1 year (χ^2 = 4.538, *P* = 0.027), 2 years (χ^2 = 5.452, *P* = 0.026) and 3 years (χ^2 = 5.467, *P* = 0.028). There were also significant differences in HE occurrence between groups C and D at 1 mo (χ^2 = 14.673, *P* = 0.014), 3 mo (χ^2 = 17.478, *P* = 0.009), 6 mo (χ^2 = 13.957, *P* = 0.011), 1 year (χ^2 = 14.576, *P* = 0.014), 2 years (χ^2 = 11.476, *P* = 0.013) and 3 years (χ^2 = 8.473, *P* = 0.017) (Figure 5).

The incidence of HE in the four groups showed a downward trend. After drug treatment, the symptoms disappeared in patients with covert and grade II HE. In patients with grade III or IV HE, the symptoms disappeared after shunt reduction, but five patients who underwent shunt reduction still had hepatic myelopathy (Table 4).

During 3 years' follow-up, 262 patients in group A, 234 in group B, 189 in group C and 160 in group D were lost to follow-up. Total survival rates were no different compared groups A with B (χ^2 = 3.376, *P* = 0.369, log-rank test), but there were significant differences between groups C and D (χ^2 =13.582, *P* = 0.014, log-rank test) (Figure 6).

The 3-mo, 6-mo, and 1-, 2-, and 3-year survival rates were different between groups A and B (χ^2 = 5.368, *P* = 0.425; χ^2 = 4.557, *P* = 0.436; χ^2 = 4.562, *P* = 0.427, χ^2 = 5.487, *P* = 0.382, and χ^2 = 4.582, *P* = 0.375, respectively); and significantly different between groups C and D (χ^2 = 13.364, *P* = 0.012; χ^2 = 12.463, *P* = 0.013; χ^2 = 12.568, *P* = 0.016; χ^2 = 11.467, *P* = 0.017, and χ^2 = 10.367, *P* = 0.027, respectively). Four hundred

Table 4 Hepatic encephalopathy occurrence in the four groups

Time	Group	HE occurrence		Occurrence rate (%)	χ^2	*P* value
		Yes	No			
1 mo	A	43	436	8.97	6.463	0.027
	B	67	345	16.26		
	C	27	190	12.44	14.673	0.014
	D	41	131	23.83		
3 mo	A	45	434	9.39	5.368	0.023
	B	79	333	19.17		
	C	39	178	17.97	17.478	0.009
	D	83	89	48.25		
6 mo	A	39	440	8.14	6.473	0.017
	B	72	340	17.47		
	C	31	186	14.28	13.957	0.011
	D	74	98	43.02		
1 year	A	36	443	7.51	4.538	0.027
	B	61	351	14.8		
	C	29	188	13.36	14.576	0.014
	D	69	103	40.11		
2 year	A	34	445	7.09	5.452	0.026
	B	49	363	11.89		
	C	23	194	10.59	11.476	0.013
	D	54	118	31.39		
3 year	A	29	443	6.14	5.467	0.028
	B	43	369	10.43		
	C	17	155	9.88	8.473	0.017
	D	42	130	24.41		
Total HE rate	A	46	433	9.6	7.932	0.016
	B	67	158	16.26		
	C	47	170	21.65	13.637	0.007
	D	69	103	40.11		

There were significant differences in incidence of hepatic encephalopathy in group A compared with groups B–D at 1, 3, 6 and 9 mo, and 1, 2 and 3 years ($P < 0.05$). HE: Hepatic encephalopathy.

and forty-nine patients died of multiorgan failure, 127 of hepatic tumor, and 298 of other causes (Table 5).

DISCUSSION

During the TIPS procedure, measuring PSG is an important step because reducing PSG can achieve good clinical results, in which a conduit is constructed within the liver between the systemic venous and portal systems, with the aim of decreasing portal systemic pressure[14]. However, too low portal pressure can lead to some complications, and to avoid the recurrence of bleeding and uncontrolled ascites induced by excess reduction of portal vein pressure, appropriate PSG levels are required[15].

Most guidelines recommend[16] that the upper threshold of the post-TIPS PSG for a patient with variceal bleeding is < 12 mmHg or 50% of baseline, and the AASLD practice guidelines suggest a

Table 5 Survival at 3 and 6 mo, and 1, 2 and 3 years

Time	Group	Survival		Survival rate (%)	χ^2	*P* value
		Yes	No			
3 mo	A	470	9	98.12	5.368	0.425
	B	401	11	97.33		
	C	184	33	84.79	13.364	0.012
	D	127	45	73.83		
6 mo	A	442	37	92.27	4.557	0.436
	B	381	31	92.47		
	C	162	55	74.65	12.463	0.013
	D	107	65	62.2		
1 year	A	397	82	82.88	4.562	0.427
	B	335	77	81.31		
	C	117	100	53.91	12.568	0.016
	D	65	107	37.79		
2 year	A	293	186	61.16	5.487	0.382
	B	245	167	59.46		
	C	59	158	27.18	11.467	0.017
	D	26	146	15.11		
3 year	A	229	250	47.8	4.582	0.375
	B	193	219	46.84		
	C	32	185	14.74	10.367	0.027
	D	16	156	9.3		
Total survival rate	A	217	262	45.3	3.376	0.369
	B	178	234	43.2		
	C	28	189	12.9	13.582	0.014
	D	12	160	6.97		

Survival rates showed no significant differences between groups A and B ($P > 0.05$), but there were significant differences between groups C and D.

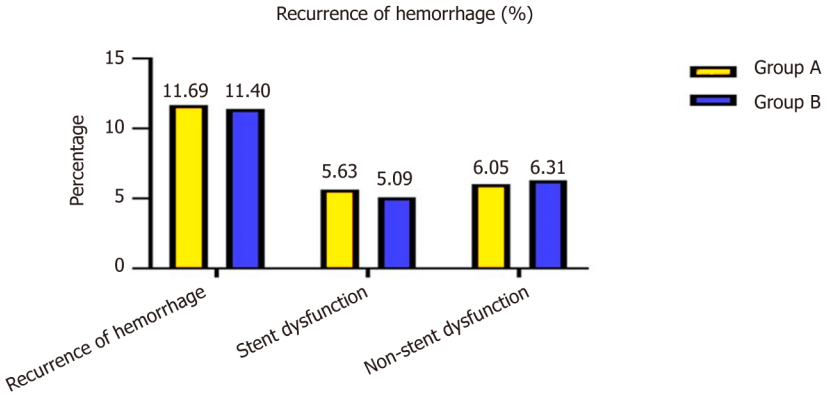
gradient of ≤ 8 mmHg[17]. Most centers presently use the thresholds for TIPS procedures.

The complications of TIPS are classified as related to the TIPS procedure itself, the stent, portosystemic shunting, *etc.*[18]. Among them, HE and deterioration of liver function, as complications related to portosystemic shunting, are associated with reduced PSG. Some of the patients with low PSG after TIPS have complications such as worsening of HE, which causes multiple admissions to hospital and increased liver enzymes and bilirubin, even though they are ultimately medically controlled[19].

This creates a paradoxical dilemma in which low PSG results in complications such as severe HE or liver function failure, and inappropriate reduction of PSG also leads to recurrence of symptoms of portal hypertension, such as variceal bleeding and ascites. The current concept of small balloon expansion is intended to reduce PSG appropriately to reduce portal hypertension without associated serious complications[20].

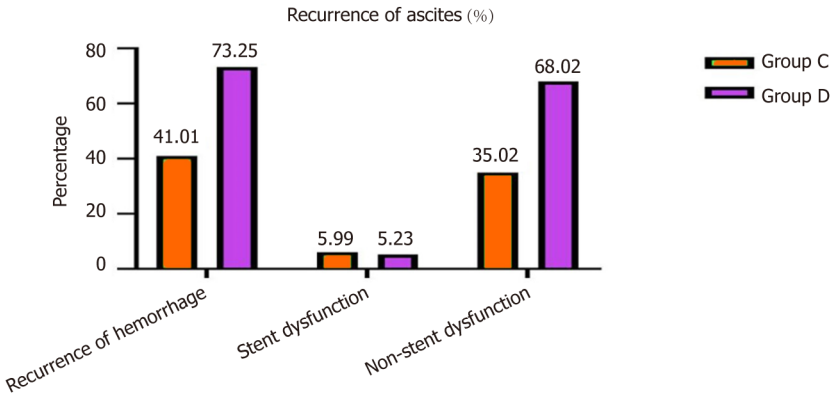
Self-expandable stents may continue to dilate until achieving their nominal diameter[21]. This means that if PSG is 11 mmHg after dilating a 10-mm stent to only 8 mm, the stent may continue to self-dilate until reaching approximately 10 mm in diameter, leading to a further decrease in PSG and an increased risk of HE. How frequently this spontaneous expansion is clinically relevant is a matter of debate, but certainly represents a limitation. This led to a further technical improvement, the controlled-expansion stents, that cannot spontaneously dilate over preset limits. More crucially, the exact reduction of PSG is unknown.

However, we do not have an answer for simple questions such as how large the shunt should be, or what PSG reduction should be targeted to prevent recurrent bleeding or ascites during TIPS. PSG should be decreased to ≤ 12 mmHg, or by > 50% of baseline (which in most cases means < 12 mmHg) to



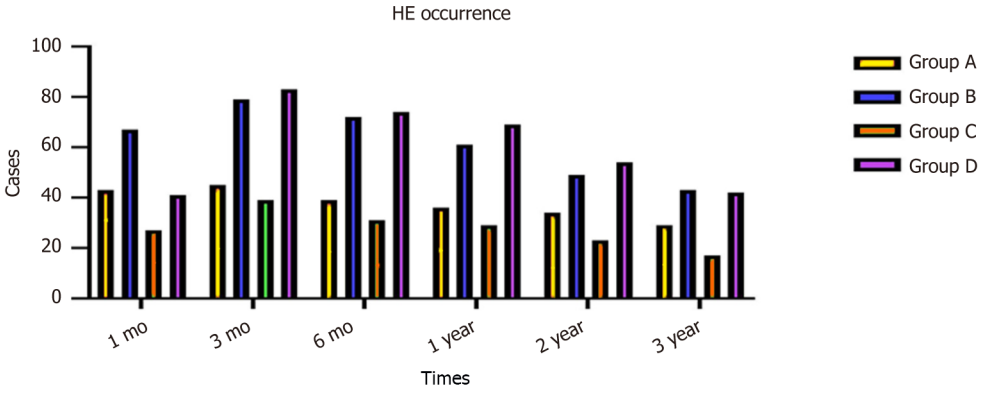
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Figure 3 Recurrent variceal bleeding after transjugular intrahepatic portosytemic shunt. Patients in groups A and B had recurrent variceal bleeding, and there was no significant difference between the groups.



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Figure 4 Recurrent ascites after transjugular intrahepatic portosytemic shunt of groups C and D. Patients in groups C and D had recurrent ascites, with a significant difference between the groups.

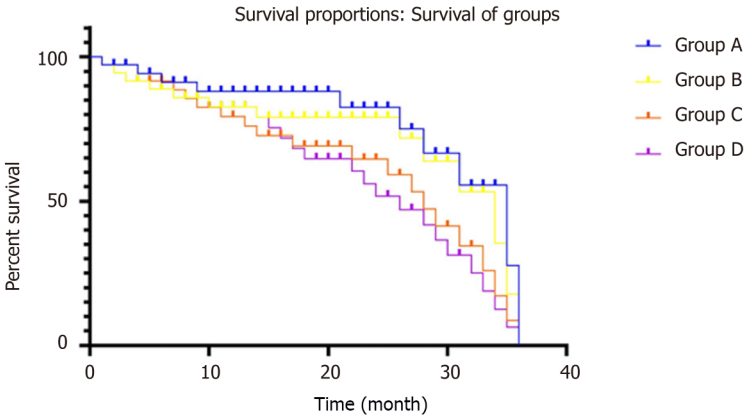


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Figure 5 Incidence of hepatic encephalopathy after transjugular intrahepatic portosytemic shunt. The incidence of HE differed significantly in group A compared with group B, and in group C compared with group D at different times. HE: Hepatic encephalopathy.

prevent the complications of portal hypertension[22]. This comes from observations that recurrent bleeding and ascites occurred almost exclusively when patients had a PSG of at least 12 mmHg after TIPS[23].

A study has suggested that despite a traditional PSG reduction to below 12 mmHg or > 50% of baseline, a PSG decrease to 14 mmHg or > 30% of baseline would be appropriate when uncovered stents are used[24]. This goal is achieved in a large proportion of patients with small diameter TIPS such as 7



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Figure 6 The survival rates of four groups. The total survival rates were not different between groups A and B, but differed significantly between groups C and D.

or 6 mm dilated shunts that are likely to result in less worsening of portosystemic shunting and hence a lower likelihood of severe HE and post-TIPS liver failure[25]. Of note, in high-risk situations, such as refractory ascites, the recent EASL guidelines recommended small diameter TIPS, although not as small as 6 mm[15].

It has been shown that complementing a small diameter TIPS with drugs can be converted into a satisfactory one by adding propranolol, even at low doses[26]. The synergistic effect of combining two different mechanisms decreases PSG by bypassing liver resistance to portal flow, and propranolol decreases PSG by reducing splanchnic blood flow. This approach achieves good clinical results and lower incidence of HE. Post-TIPS HE is a complex condition that is determined by TIPS diameter and many nonhemodynamic factors. Age, degree of liver and kidney failure, chronic inflammation, urease-producing intestinal bacteria, bacterial translocation and malnutrition/sarcopenia, are other important factors that can modulate the therapeutic effect[27]. Several of them are associated with post-TIPS HE and survival[28]. Therefore, appropriate reduction of PSG can reduce the occurrence of TIPS-related complications, such as HE. Combined with drugs, if good clinical results are achieved, it is not necessary to reduce PSG too low to produce higher TIPS-related complications.

Based on the above, we hypothesize that for patients with gastrointestinal bleeding and refractory ascites requiring TIPS, one-third reduction of PSG of the baseline is appropriate, which was supplemented by drug-lowering portal pressure therapy. We should reduce portal hypertension as much as possible, achieve good therapeutic results, and minimize complications, especially the incidence of HE and compromise of liver function.

In this study, we divided the patients with gastrointestinal bleeding and refractory ascites who required TIPS into four groups, to make a detailed evaluation of the clinical effects. The results showed that, as for patients with variceal bleeding who required TIPS placement, PSG reduced by one third compared with < 12 mmHg baseline, the two groups had a similar effect on variceal bleeding, but the incidence of HE and compromise of liver function differed. During the TIPS procedure, to achieve the goal of reducing PSG by one third, a small balloon was required for gradual dilatation, slowly from 6 mm to 8 mm, which would also be useful for the controlled expansion stent that has been introduced in clinical practice. During the dilatation process, the operating procedure will be slower, PSG measurement will take approximately 30 min longer, and two more balloons will be used, resulting in increased cost. However, the cost should be worthwhile in comparison to the cost of complications.

For patients with refractory ascites who required TIPS, the incidence of HE and compromise of liver function were obviously different. In the short term, the symptoms of ascites disappear or subside, but in the medium and long term, PSG drops less, ascites still recurs in some cases, and drug therapy is necessary. Post-TIPS HE is a complex condition that is determined by TIPS and many nonhemodynamic factors. The liver function reserve of patients with refractory ascites and survival rate are poor, and the patients are prone to hypoproteinemia and electrolyte disturbances, which are likely to cause recurrence of ascites and require drug treatment[29]. In this circumstance, to reduce TIPS-related complications and liver function damage, it is not necessary to reduce PSG drastically, one-third PSG reduction plus drug therapy would be appropriate.

As a retrospective analysis, our study may manifestaed some limitations. For the one, it maybe need randomized controlled trials to validate the results. Next, to achieve main goal of reducing the PSG by one third, it requires gradual balloon dilatation from 6 mm to 8 mm, the operating procedure will be slower, PSG measurement will take approximately 30 min longer, and two more balloons will be used, resulting in increased cost. Finally, our hypothesis needs to be validated by a comparative study on the results of small balloon dilatation.

CONCLUSION

This multicenter retrospective study showed that patients who underwent TIPS creation with PSG reduced to one third of baseline or to < 12 mmHg or 50% of baseline had similar successful clinical outcomes. However, PSG reduced to < 12 mmHg had a lower rate of HE and liver compromise. Given that the PSG will become more controllable in the future with the advent of controllable stents, we believe that our concept is worthy of clinical application.

ARTICLE HIGHLIGHTS

Research background

Transjugular intrahepatic portosystemic shunt (TIPS) is placed important role in the therapy of complications of liver cirrhosis. Measuring portosystemic pressure gradient (PSG) is important during the TIPS procedure. Reducing PSG can achieve good clinical results, but when PSG is too low, TIPS leads to many complications. Factors associated with post-TIPS complications depend mainly on portocaval pressure gradient and the volume of blood shunted through the liver. Several guidelines recommend that PSG reduced to 12 mmHg after TIPS creation achieves better clinical outcomes. However, in that situation, the incidence of hepatic encephalopathy (HE) was higher in clinical practice. There is still no suitable criterion for a reduction in PSG, which can both reduce PSG and maximize clinical results and minimize HE, and few data are available to calculate an appropriate PSG value.

Research motivation

We report our multicenter retrospective study to compare the rate of HE and clinical results of reducing PSG by one third of baseline with PSG reduction to < 12 mmHg in patients with portal hypertension who required TIPS placement wtih of variceal bleeding and ascites.

Research objectives

The main objective was to establish that patients who underwent TIPS PSG reduced by one third of baseline compared with PSG reduced to < 12 mmHg of baseline were associated with similar successful clinical outcomes.

Research methods

We hypothesized that reducing PSG by one third of baseline compared with < 12 mmHg of baseline would result in a lower rate of HE and liver compromise. The patients were divided into four groups: Group A (variceal hemorrhage and PSG reduced by one third, n = 479); group B (variceal hemorrhage and PSG reduced to < 12 mmHg, n = 412); group C (refractory ascites and PSG reduced by one third, n = 217); and group D (refractory ascites and PSG reduced to < 12 mmHg, plus medication, n = 172). The clinical outcomes were compared and evaluated. Data measurements results of the four groups were normally distributed, and expressed as mean ± standard deviation, and their differences were determined using t-test. The statistical analyses were performed with SPSS version 22.0.

Research results

This study showed that during TIPS placement, when PSG was reduced by one third compared with < 12 mmHg of baseline, recurrent bleeding showed no significant difference, but recurrent ascites did differ significantly. The probability of total hepatic impairment within 3 years was significantly different. During follow-up, the total incidence of HE differed significantly. The total survival rates were no different for the variceal bleeding patients but were significantly different for the patients with refractory ascites.

Research conclusions

We found that patients who underwent TIPS PSG reduced by one third of baseline compared with reduced to < 12 mmHg of baseline were associated with similar successful clinical outcomes, but PSG reduced by one third resulted in a lower rate of HE and liver compromise.

Research perspectives

Measuring PSG is important during the TIPS procedure. Reducing PSG can achieve good clinical results, but when PSG is too low, TIPS leads to many complications. Reduction of PSG by one third of baseline is recommended to decrease the probability of liver function impairment after TIPS, decrease the incidence of HE, and increase survival in patients with refractory ascites.

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FOOTNOTES

Author contributions: Chu JG designed the research; Luo SH and Zhou MM performed the research; Han SL, Zhang XQ analyzed the data; Luo SH wrote the paper; Cai MJ revised the paper; All authors have read and approved the final version to be submitted.

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ORIGINAL ARTICLE

Retrospective Study

Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection as a salvage option for recurrent achalasia

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Abstract

BACKGROUND

For recurrent achalasia after initial peroral endoscopic myotomy (POEM) failure, repeat POEM (Re-POEM) has been reported as a treatment option. However, severe esophageal interlayer adhesions caused by previous procedures impede the successful establishment of a submucosal tunnel and lead to aborted Re-POEM procedures. Our team previously described POEM with simultaneous submucosal and muscle dissection (POEM-SSMD) as a feasible solution for achalasia with severe interlayer adhesions.

AIM

To investigate the effectiveness and safety of Re-POEM with simultaneous submucosal and muscle dissection (Re-POEM-SSMD).

METHODS

A total of 1049 patients with achalasia who underwent successful endoscopic myotomy at the Digestive Endoscopic Center of Chinese PLA General Hospital from December 2014 to May 2022 were reviewed. Patients with recurrent achalasia who experienced initial POEM clinical failure were retrospectively included in this study. The primary endpoint was retreatment clinical success, defined as an Eckardt score ≤ 3 during the postretreatment follow-up and no need for additional treatment. Procedure-related adverse events, changes in manometric lower esophageal sphincter (LES) pressure and reflux complications, as well as procedure-related parameters, were recorded.

RESULTS

Sixteen patients underwent Re-POEM (9 patients) or Re-POEM-SSMD (7 patients)

successfully at a median of 45.5 mo (range, 4-95 mo) after initial POEM. During a median follow-up period of 31 mo (range, 7-96 mo), clinical success (Eckardt score ≤ 3) was achieved in 8 (88.9%) and 6 (85.7%) patients after Re-POEM and Re-POEM-SSMD, respectively (P = 0.849). The median Eckardt score dropped from 4 (range, 3-8) at preretreatment to 1 (range, 0-5) at postretreatment in the Re-POEM group (P = 0.025) and from 5 (range, 2-8) to 2 (range, 0-4) in the Re-POEM-SSMD group (P < 0.001). The mean manometric LES pressure decreased from 23.78 ± 9.04 mmHg to 11.45 ± 5.37 mmHg after Re-POEM (P < 0.001) and from 26.80 ± 7.48 mmHg to 11.05 ± 4.38 mmHg after Re-POEM-SSMD (P < 0.001). No serious adverse events were recorded in both groups.

CONCLUSION

In conclusion, Re-POEM-SSMD appears to be a safe and effective salvage therapy for recurrent achalasia with severe interlayer adhesions.

Key Words: Esophageal achalasia; Recurrence; Peroral endoscopic myotomy; Simultaneous submucosal and muscle dissection; Interlayer adhesion; Salvage therapy

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Core Tip: For recurrent achalasia after initial peroral endoscopic myotomy (POEM) failure, repeat POEM (Re-POEM) has been reported as a potential treatment option. However, severe interlayer adhesions caused by previous procedures impede the successful establishment of a submucosal tunnel. In this study, we retrospectively analyzed the data of patients who had persistent or recurrent symptoms after initial POEM and underwent Re-POEM or Re-POEM with simultaneous submucosal and muscle dissection (Re-POEM-SSMD). The results show that Re-POEM-SSMD could serve as an effective and safe salvage option for patients with recurrent achalasia when conventional POEM procedures are impeded by severe interlayer adhesions.

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INTRODUCTION

Achalasia is an uncommon esophageal motor disorder estimated to affect 2 to 3 people per 100000 people in the general population and is caused by failure of adequate relaxation of the lower esophageal sphincter (LES) and impaired esophageal peristalsis[1]. Treatment options mainly include endoscopic botulinum toxin injection, laparoscopic Heller's myotomy (LHM), pneumatic dilation (PD) and peroral endoscopic myotomy (POEM) and aim to relax or cause a mechanical disruption of the LES in order to achieve symptom relief and restore quality of life[2].

POEM is a super minimally invasive surgery (SMIS) with transesophageal tunnelling endoscopic myotomy and has been used to treat achalasia since 2010[3]. Because of digestive endoscopic tunnel techniques (DETTs), diseases such as achalasia that were previously treated surgically can now be treated endoscopically by creating a submucosal tunnel as a surgical space[4]. Based on results from a multitude of retrospective studies, the clinical success rate of POEM for achalasia treatment exceeds 90%, with only approximately 5%-10% of patients remaining symptomatic postoperatively[5-7].

Recently, repeat POEM (Re-POEM) has been reported as an endoscopic therapeutic option for patients with persistent or recurrent symptoms of achalasia after POEM[8,9]. However, severe interlayer adhesions in the submucosa caused by previous POEM could hinder the establishment of a submucosal tunnel and therefore increase the procedural difficulty of RE-POEM. Coincidentally, we previously reported POEM with simultaneous submucosal and muscle dissection (POEM-SSMD) as a treatment option for patients with achalasia and severe interlayer adhesions[10]. Herein, we retrospectively compared Re-POEM and Re-POEM with simultaneous submucosal and muscle dissection (Re-POEM-SSMD) as a salvage therapy for patients with prior failed POEM procedures in terms of clinical efficacy and safety.

MATERIALS AND METHODS

Patients

We reviewed 1049 patients with achalasia who underwent successful endoscopic myotomy (POEM, Re-POEM and Re-POEM-SSMD) at the Digestive Endoscopic Center of Chinese PLA General Hospital from December 2014 to May 2022. Patients with recurrent acha-lasia who experienced initial POEM clinical failure were retrospectively included in this study. Post-POEM clinical failure was defined as having persistent or recurrent symptoms for at least 3 mo. The diagnosis of recurrent achalasia was based on medical history, clinical symptoms, esophagogastroduodenoscopy (EGD), barium swallow and manometry. Patients who had undergone surgical therapy for achalasia or had previous surgery of the esophagus were excluded. Patient demographics, baseline clinical status and preprocedural evaluation were recorded. The esophageal symptoms and clinical status were quantified using the Eckardt score [11]. The study was reviewed and approved by the Ethics Committee of Chinese PLA General Hospital (Approval No. S2021-207-01). All study participants signed a written informed consent form.

Interventions

The standard endoscopic procedures were performed by two senior endoscopists with sufficient experience in therapeutic endoscopy as previously described[12]. Patients were kept in the supine position with the right shoulder elevated. Re-POEM or Re-POEM-SSMD was performed using a single-channel high-definition gastroscope and carbon dioxide (CO_2) gas for insufflation during the procedure.

During the Re-POEM procedure, the scarred area was avoided in the opposite orientation of the initial POEM site to allow the establishment of a new submucosal tunnel. When severe interlayer adhesions impeded the establishment of new submucosal tunnel, Re-POEM-SSMD was performed to dissect the submucosa and muscle synchronously as we described previously[13].

The sequential steps were generally performed as follows: (1) The submucosal space was created by injecting methylene blue saline solution to create a liquid mat, and then an inverse T incision was made for tunnel entry; (2) the extended submucosal tunnel was terminated at the lesser curvature of the cardia (Re-POEM) or as far as possible due to the interlayer adhesions (RE-POEM-SSMD); (3) a circumferential layer myotomy was performed at the proximal esophagus and a full-thickness myotomy was performed extended to 2-3 cm below the cardia (Re-POEM), or the submucosa and muscle were incised simultaneously and a full-thickness myotomy was performed 2-3 cm below the cardia without impairing the mucosa (RE-POEM-SSMD); and (4) then the mucosal incision site was sealed using endoscopic metal clips after hemostasis was achieved.

All patients were observed for gas-related adverse events such as subcutaneous emphysema, pneumothorax or pneumoperitoneum to confirm the absence of gas leakage. After the procedure, dietary restrictions were enforced, and proton pump inhibitors (PPIs) and antibiotics were routinely administered intravenously. Upon discharge, patients were instructed to continue taking oral PPIs and adhere to a soft diet for at least four weeks.

Outcome measures

The primary endpoint was retreatment clinical success, which was defined as an Eckardt score of ≤ 3 during the postprocedure follow-up and no need for additional treatment. Secondary outcome measures were procedure-related adverse events, changes in manometric LES pressure and Eckardt score, reflux complications after retreatment, and procedure-related parameters such as operative time, tunnel length, myotomy length and length of hospital stay.

High-resolution manometry and Ling classification

LES pressures before and after retreatment were recorded by using a high-resolution manometry (HRM) system at 1-cm intervals with high-fidelity circumferential sensors (ManoScan; Sierra Scientific Instruments Inc, Los Angeles, CA, United States) as we previously described[12]. HRM was performed with patients in a supine position after a minimum of 6 hours of fasting. Pressure data of 10 wet swallows were recorded and analyzed with Mano-View software. Ling classification of achalasia, which is used to describe the endoscopic morphology of the esophageal lumen, was recorded as we previously described[14].

Follow-up

Upper gastrointestinal endoscopic evaluation including monitoring of wound healing and examination of objective signs of reflux esophagitis and HRM were scheduled at 3, 6 and 12 mo post retreatment. Patient-reported outcomes such as Eckardt scores and symptomatic reflux were recorded in the outpatient clinic or by telephone at 3, 6 and 12 mo and then annually thereafter.

Statistical analysis

The results were expressed as the mean with a standard deviation (SD) or median with a range for quantitative data and numbers with percentages for categorical data. Student's *t* test and the Mann–Whitney *U* test were used to compare baseline characteristics (other than sex), operation

Table 1 Demography and clinical profile of patients with recurrent achalasia after initial peroral endoscopic myotomy

	Total (*n* = 16)	Re-POEM (*n* = 9)	Re-POEM-SSMD (*n* = 7)	*P* value
Age, mean ± SD, yr	43.4 ± 16.3	43.0 ± 15.4	43.86 ± 18.7	0.916
Male, *n* (%)	9 (56.3)	4 (44.4)	5 (71.4)	0.008
Duration of dysphagia, median (range), mo	86.5 (16-420)	92 (16-147)	24 (16-420)	0.751
Time symptoms recurred after initial POEM, median (range), mo	15.5 (0-92)	12 (0-92)	7.5 (0-50)	0.414
Interval between initial POEM and Re-POEM, median (range), mo	45.5 (4-95)	64 (14-95)	9 (4-60)	0.013
Achalasia endoscopy type, *n* (%)				
Ling I	2 (12.5)	2 (22.2)	0 (0)	
Ling IIa	5 (31.3)	3 (33.3)	2 (28.6)	
Ling IIb	1 (6.3)	0 (0)	1 (14.3)	
Ling IIc	5 (31.3)	3 (33.3)	2 (28.6)	
Ling IIIr	3 (18.8)	1 (11.1)	2 (28.6)	

The values are expressed as mean ± SD or medians (range) for quantitative data, and *n* (%) for categorical data. SD: Standard deviation; POEM: Peroral endoscopic myotomy; Re-POEM: Repeat peroral endoscopic myotomy; Re-POEM-SSMD: Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection.

parameters, Eckardt score and LES pressure between the Re-POEM and Re-POEM-SSMD groups. Fisher's exact test was used to compare sex, clinical success and reflux complications between the Re-POEM and Re-POEM-SSMD groups. The Friedman Test was used to compare the baseline, preretreatment and postretreatment Eckardt score and Paired Student's *t* test was used to compare preretreatment and postretreatment LES pressure. All reported *P* values were two-tailed, with statistical significance set at $P < 0.05$. Statistical analyses were performed using SPSS software (version 26.0; SPSS, Chicago, IL, United States).

RESULTS

Clinical characteristics of patients

Sixteen patients (9 male, 7 female; mean age 43.4 ± 16.3 years) who experienced initial POEM clinical failure were diagnosed with recurrent achalasia. The median duration of dysphagia was 86.5 mo (range, 16-420 mo). Symptoms of achalasia recurred a median time of 15.5 mo (range, 0-92 mo) after initial POEM. Retreatment procedures were conducted after a mean of 45.5 mo (range, 4-95 mo) following the initial POEM. According to the Ling classification, 2 patients (12.5%), 5 patients (31.3%), 1 patient (6.3%), 5 patients (31.3%) and 3 patients (18.8%) had Ling I, Ling IIa, Ling IIb, Ling IIc and Ling IIIr types, respectively (Table 1).

Procedure-related parameters and adverse events

Re-POEM (9 patients) and Re-POEM-SSMD (7 patients) procedures were conducted successfully in all patients. A representative case of Re-POEM-SSMD is described in Figure 1. The mean operative time was 39.22 ± 15.19 min in the Re-POEM group and 47.86 ± 9.71 min in the Re-POEM-SSMD group ($P = 0.414$). The median lengths of the submucosal tunnel and myotomy were 9 cm (range, 7-13 cm) and 6 cm (range, 4.5-11 cm) in the Re-POEM group and 5 cm (range, 4-12 cm) ($P = 0.012$) and 6 cm (range, 4-11 cm) ($P = 1.000$) in the Re-POEM-SSMD group, respectively. Prophylactic antibiotics were administered for 3 to 6 d after the procedures. The average length of hospital stay was 6.0 ± 1.7 d and 7.1 ± 1.3 d after Re-POEM and Re-POEM-SSMD, respectively ($P = 0.454$). Regarding procedure-related adverse events, computed tomography showed pneumoperitoneum in two patients in the Re-POEM group but no apparent clinical consequence occurred during later follow-up. One patient in the Re-POEM-SSMD group sustained mucosal perforation during submucosal tunnel construction and was treated by endoscopic metal clips, no additional clinical complications were observed (Table 2).

Treatment outcomes and reflux complications

During a median follow-up period of 31 mo (range, 7-96 mo), clinical success (Eckardt score ≤ 3) was achieved in 8 (88.9%) and 6 (85.7%) patients after Re-POEM and Re-POEM-SSMD, respectively ($P =$

Table 2 Operation information and treatment outcomes of patients with recurrent achalasia after initial peroral endoscopic myotomy

	Re-POEM (*n* = 9)	Re-POEM-SSMD (*n* = 7)	*P* value
Operation parameters			
Operative time, mean ± SD, min	39.22 ± 15.19	47.86 ± 9.71	0.414
Submucosal tunnel length, median (range), cm	9 (7-13)	5 (4-12)	0.012
Myotomy length, median (range), cm	6 (4.5-11)	6 (4-11)	1.000
Postoperative hospital stay, mean ± SD, d	6.0 ± 1.7	7.1 ± 1.3	0.454
Adverse events, *n*			
Mucosal injury	0	1	
Subcutaneous emphysema	0	0	
Pneumothorax	0	0	
Pneumoperitoneum	2	0	
Tunnel infection	0	0	
Clinical success during follow-up, *n* (%)	8 (88.9)	6 (85.7)	0.849
Eckardt score, median (range)			
Pre-POEM	6 (2-8)	5 (2-8)	0.892
Preretreatment	4 (3–8)	5 (2-8)	0.425
Postretreatment	1 (0-5)	2 (0-4)	0.416
Lower esophageal sphincter pressure, mean ± SD, mmHg			
Preretreatment	23.78 ± 9.04	26.80 ± 7.48	0.489
Postretreatment	11.45 ± 5.37	11.05 ± 4.38	0.872
Reflux complications			
Symptomatic reflux, *n* (%)	6 (66.7)	0 (0)	0.001
R, *n* (%)	6 (66.7)	3 (42.9)	0.100

SD: Standard deviation; POEM: Peroral endoscopic myotomy; Re-POEM: Repeat peroral endoscopic myotomy; Re-POEM-SSMD: Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection.

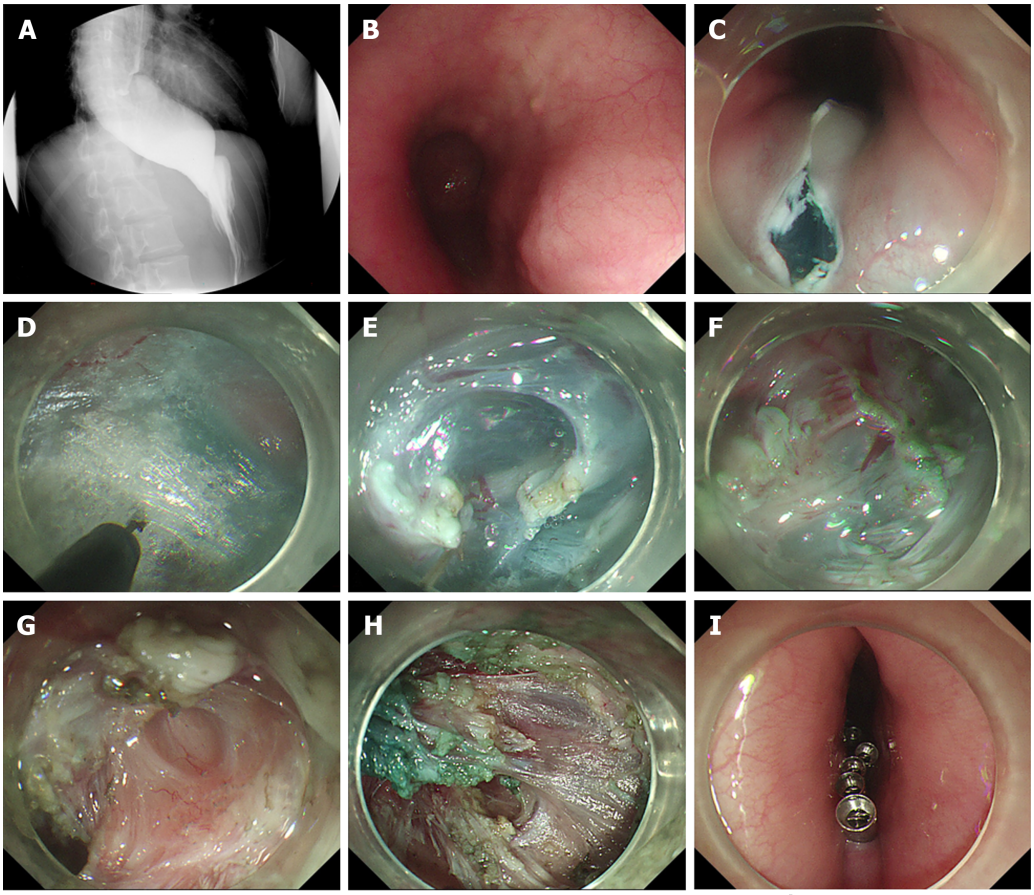
0.849). None of the patients underwent balloon dilation, temporary esophageal stent placement, or botulinum toxin injections after POEM or retreatment. One patient in the Re-POEM group had recurrent symptoms of achalasia and chose a follow-up strategy. Reintervention was performed in 1 patient in the Re-POEM-SSMD group for recurrent symptoms (Table 2).

The median Eckardt score dropped from 4 (range, 3-8) at preretreatment to 1 (range, 0-5) at postretreatment in the Re-POEM group ($P = 0.025$) and from 5 (range, 2-8) to 2 (range, 0-4) in the Re-POEM-SSMD group ($P < 0.001$) (Figure 2). The mean manometric LES pressure decreased from 23.78 ± 9.04 mmHg to 11.45 ± 5.37 mmHg after Re-POEM ($P < 0.001$) and from 26.80 ± 7.48 mmHg to 11.05 ± 4.38 mmHg after Re-POEM-SSMD ($P < 0.001$) (Figure 3).

At the follow-up EGD, no severe complications, such as delayed bleeding or mucosal barrier failure, were detected. The overall rate of clinical reflux complications following retreatment was 56.3% (9/16). Six patients developed symptomatic gastroesophageal reflux such as heartburn and reflux after Re-POEM and were treated by intermittent administration of standard-dose PPI. Reflux esophagitis was detected in six patients (Los Angeles classification A, 2; B, 1; C, 3) in the Re-POEM and three (Los Angeles classification B, 1; C, 2) in the Re-POEM-SSMD group ($P = 0.100$).

DISCUSSION

Although POEM is highly effective as a first-line treatment for achalasia, a small percentage of patients remain symptomatic or experience relapse after POEM[15,16]. Inadequate myotomy below the EGJ, premature fusion healing and early myotomy site scarring may be the main causes of recurrent dysphagia in POEM patients. Prior botulinum toxin injections and balloon dilations may also lead to substantial fibrosis, making myotomy more difficult. Consensus on the optimal management strategy



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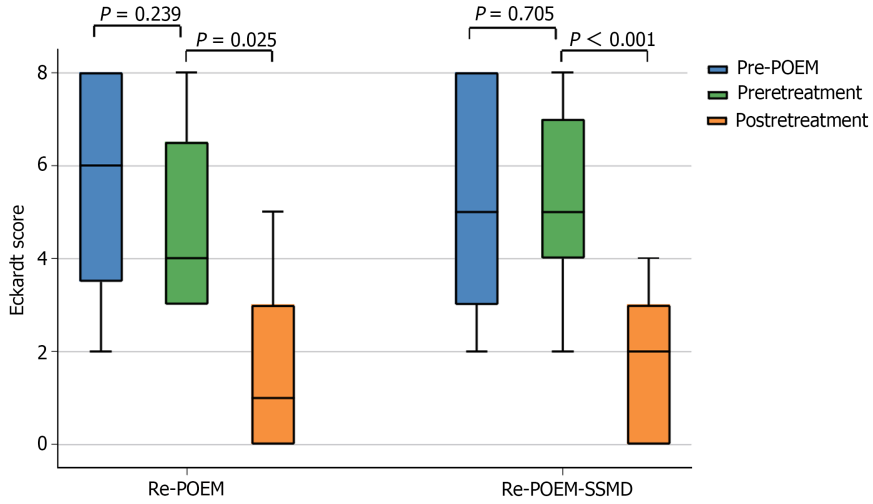
Figure 1 Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection in a 28-year-old woman with recurrence of symptoms after previous peroral endoscopic myotomy. A: Radiographic image showing the difficulty of barium to pass through the pipe into the stomach; B: The mucosal scar of previous peroral endoscopic myotomy; C: Reverse T incision of submucosal entry near the scarred area; D: Construction of the submucosal tunnel; E: Obvious submucosal fibrosis disconnected by electrocoagulation forceps; F: Simultaneous submucosal and muscle dissection started from the muscular scar; G: Full-thickness myotomy of the esophagus; H: Exact hemostasis using hemostatic forceps; I: Closure of the mucosal entry point with metal clips.

for patients with recurrent achalasia after initial POEM failure has not yet been reached.

Re-POEM after failed surgical Heller myotomy has been reported to have good short-term results[17,18]. Recently, Re-POEM has also been described as an effective endoscopic therapy for the treatment of achalasia after failed initial POEM procedures with clinical success rates ranging from 63%-100%[8,19]. Moreover, Ichkhanian *et al*[9] reported that when compared with pneumatic dilation and LHM, Re-POEM had a higher clinical success rate in the management of patients with failed initial POEM. As a SMIS, Re-POEM is less invasive compared to Heller myotomy and requires fewer attempts compared to balloon dilation. In this retrospective study, we found that Re-POEM had a clinical success rate of 88.9% for recurrent achalasia after initial POEM failure, which is similar to that reported in previous studies.

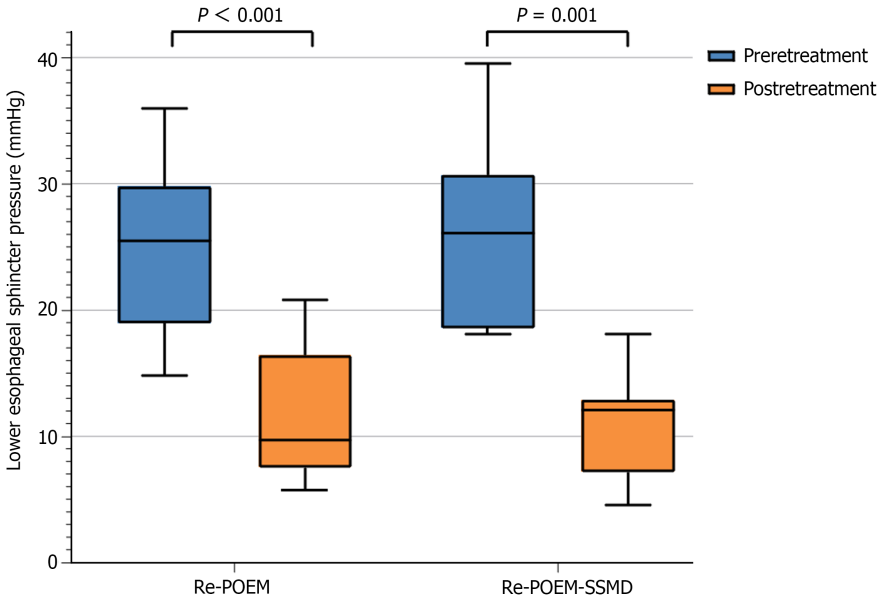
In POEM procedures, submucosal tunnel dissection and endoscopic myotomy must extend deep beyond the gastroesophageal junction (at least 2 cm) to guarantee the success of POEM[16]. Re-POEM is technically challenging due to the presence of post-POEM interlayer adhesions and fibrosis and is usually performed using a new submucosal tunnel in a relatively unobstructed area to avoid contact with the previous procedure site. The presence of severe esophageal interlayer adhesions impede the establishment of a new submucosal tunnel. In 2014, our team introduced a novel, modified procedure, entitled POEM-SSMD, to address severe interlayer adhesions[10]. As a result of the procedure, the long-term symptom relief rate was 83% with controllable adverse events for patients with achalasia and severe interlayer adhesions[13]. Interlayer adhesions are more common in patients with recurrent achalasia who underwent initial POEM than in patients with untreated achalasia. Re-POEM-SSMD can be performed in the scar area of an earlier myotomy site without considering the adhesions or fibrosis from the previous operation, which makes it easier for the endoscopist to select the preferred direction of the myotomy. Based on our data, Re-POEM-SSMD had a comparable clinical success rate of 85.7% and could serve as an effective salvage option when conventional endoscopic procedures are impeded by severe interlayer adhesions caused by previous POEM procedures.

The orientation selection of Re-POEM and whether to use SSMD operation depends on the preference of the endoscopists and their own technical levels. Before retreatment, mucosal inflammation and submucosal fibrosis of the esophagus are classified to determine the degree of adhesion[20]. If the initial



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Figure 2 Eckardt score changes in patients with recurrent achalasia. POEM: Peroral endoscopic myotomy; Re-POEM: Repeat peroral endoscopic myotomy; Re-POEM-SSMD: Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection.



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Figure 3 Lower esophageal sphincter pressure changes in patients with recurrent achalasia. Re-POEM: Repeat peroral endoscopic myotomy; Re-POEM-SSMD: Repeat peroral endoscopic myotomy with simultaneous submucosal and muscle dissection.

operative region has obvious adhesions and the rest of esophagus has no or mild adhesions, another tunnel can be created at the contralateral esophagus to improve the success rate of Re-POEM. If the esophagus has severe and extensive adhesions and performing SSMD is necessary, the endoscopist can choose to create a tunnel at the original procedure site, which usually means a relatively comfortable operative orientation and may help to improve the technical success rate.

In the Re-POEM-SSMD group, due to the presence of post-POEM adhesions and fibrosis, the submucosa and muscle layer are cut simultaneously from the adhesion site with aborted intended termination point of the submucosal tunnel, leading to a shorter submucosal tunnel than that created in conventional RE-POEM procedures.

The incidence of major adverse events such as vital-sign instability and intensive care unit stay ranged between 2% and 4% in the published literature[21-23]. No major adverse events were recorded in both groups of our study. A rupture in the mucosa may compromise its integrity and lead to esophageal leakage and secondary infection, which should be managed appropriately once identified. One mucosal injury in the Re-POEM-SSMD group was successfully managed with secure closure and sufficient drainage without the need for surgical repair. The incision made at the tunnel entry site in the mucosa should be tightly clipped after myotomy. CO_2 should be used as the insufflation gas and a lower

flow should be used sparingly once the creation of the submucosal tunnel or SSMD is started. In our study, two cases of pneumoperitoneum in the Re-POEM group were recorded but no apparent clinical consequence occurred during later follow-up. It should be noted that Re-POEM-SSMD should be performed by endoscopists with sufficient experience in therapeutic endoscopy and after preparation for all contingencies.

Gastroesophageal reflux and Barrett's esophagus are usually considered long-term major concerns after POEM with no antireflux procedure[15]. Our study showed that fewer cases of symptomatic reflux occurred in the Re-POEM-SSMD group than in the Re-POEM group. Re-POEM-SSMD was more likely to be performed in the earlier myotomy site, while Re-POEM was usually performed in the opposite direction with a new myotomy site. Therefore, in Re-POEM-SSMD, less damage to the muscular layer may be responsible for less symptomatic reflux. Nevertheless, follow-up EGD showed no difference in postretreatment LES pressure or incidence of esophagitis between the two groups. Therefore, the results should be interpreted with caution and effectiveness of Re-POEM-SSMD in reducing reflux must be verified in prospective studies with large samples.

There are several limitations to the current study. For instance, a small sample of patients were classified into two groups, so a subgroup analysis of the Ling classification could not be performed. Moreover, the retrospective nature of the study and variable duration of follow-up were also limitations. There was also a potential for patient selection bias.

CONCLUSION

In conclusion, Re-POEM-SSMD appears to be a safe and effective salvage option for recurrent achalasia with adhesions or fibrosis caused by previous POEM procedures, thereby broadening the applicability of Re-POEM to patients after failed POEM. We are awaiting the results of the RCT and long-term follow-up.

ARTICLE HIGHLIGHTS

Research background

For recurrent achalasia after initial peroral endoscopic myotomy (POEM) failure, repeat POEM (Re-POEM) has been reported as a treatment option.

Research motivation

Interlayer adhesions caused by previous POEM procedures are common, which could impede the establishment of a submucosal tunnel and even lead to aborted Re-POEM procedures.

Research objectives

This study aimed to evaluate the efficacy and safety of Re-POEM with simultaneous submucosal and muscle dissection (Re-POEM-SSMD) as a salvage therapy for recurrent achalasia with severe esophageal interlayer adhesions after prior failed POEM procedures.

Research methods

Patients diagnosed with recurrent achalasia who underwent Re-POEM or Re-POEM-SSMD in our center were retrospectively analyzed in terms of clinical efficacy, incidence of procedure-related adverse events and reflux complications.

Research results

Clinical success (Eckardt score ≤ 3) was achieved in 8 (88.9%) patients after Re-POEM and 6 (85.7%) patients after Re-POEM-SSMD (P = 0.849). No severe adverse events were recorded in both groups.

Research conclusions

Re-POEM-SSMD could serve as a safe and effective salvage therapy for recurrent achalasia with severe interlayer adhesions.

Research perspectives

Further studies in large-scale patient population and prospective studies are required to verify the efficacy and safety of Re-POEM-SSMD in the management of patients after failed POEM. Further evaluation of the validity of Re-POEM-SSMD in reducing reflux is also awaited.

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FOOTNOTES

Author contributions: Chai NL was the guarantor and designed the study; Lin YJ and Liu SZ participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Li LS, Han K, and Shao BZ revised the article critically for important intellectual content; Linghu EQ and Chai NL were the patient's endoscopists; and all authors read and approved the final version.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Chinese PLA General Hospital (Approval No. S2021-207-01).

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Data sharing statement: No additional data are available.

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EDITORIAL

Future therapeutic implications of new molecular mechanism of colorectal cancer

Sen Lu, Cheng-You Jia, Jian-She Yang

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Abstract

High incidence (10.2%) and mortality (9.2%) rates led to the ranking of colorectal cancer (CRC) as the second most malignant tumor spectrum worldwide in 2020. Treatment strategies are becoming highly dependent on the molecular characteristics of CRC. The classical theories accept two models depicting the origin of CRC: The progression of adenoma to cancer and transformation from serrated polyps to cancer. However, the molecular mechanism of CRC development is very complex. For instance, CRCs originating from laterally spreading tumors (LST) do not adhere to any of these models and exhibit extremely serious progression and poor outcomes. In this article, we present another possible pathway involved in CRC development, particularly from LST, with important molecular characteristics, which would facilitate the design of a novel strategy for targeted therapy.

Key Words: Colorectal cancer; Laterally spreading tumors; Molecular mechanism; Truncated adenomatous polyposis coli mutation; Golgi fragmentation; Cancerous mechanism

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Core Tip: Although laterally spreading tumors (LST) are considered vital precancerous lesions of colorectal cancer (CRC), the mechanism mediating their transition to CRC development is unclear. Adenomatous polyposis coli (APC)-truncating mutations driven by Golgi fragmentation are very important cellular events that can abrogate the microtubule binding properties of APC. This effect reduces the stability of microtubules, impacts cell proliferation and survival, causes chromosomal instability, and increases migration. Downstream characteristics of Golgi fragmentation indicate alterations in Ataxia-telangiectasia mutated and anoctamin 5 expression, whereas their gene expression changes are significant in LST. This implies a novel pathway for CRC development from LST.

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INTRODUCTION

In 2020, the World Health Organization International Agency for Research on Cancer officially released the latest cancer data, showing a 10.2% and 12.2% incidence of colorectal cancer (CRC)[1,2]. Consequently, CRC was ranked the third and second highest in malignant tumor spectrum and in China, respectively, while its mortality of 9.2% ranked it the second highest among all cancers[1,2]. Most incidences of CRC develop from benign polyps (adenomas and serrated polyps) through a series of genetic and epigenetic changes that occur over 10 to 15 years[3,4].

A laterally spreading tumor (LST) is a special digestive tract tumor that is an important precancerous lesion of CRC. Its morphological features are hidden and, consequently, it is easily misdiagnosed. LST can develop into progressive CRC within 3 years with a very poor prognosis[5]. Large-scale controlled studies have shown that LST patients have an 8.4%-52.5% possibility of developing CRC, and benign LST lesions can develop into advanced CRC within 3 years[5].

Pathologically, LST has certain similarities to colorectal polyps and adenoma, and the molecular mechanisms underlying their progression to carcinoma have been clearly elucidated. However, studies on how LST develops into CRC are rare, and the molecular mechanism of the associated carcinogenesis is unclear. Therefore, systematically and comprehensively exploring the molecular mechanisms underlying the malignant transformation of LST to CRC is critical. Specifically, the exploration would have potential theoretical significance and clinical value for early diagnosis and precise treatment of CRC. Here, we present a potential alternative pathway mediating the development of CRC, particularly from LST, with critical molecular characteristics, which would facilitate the design of a novel strategy for targeted therapy.

MECHANISM OF CRC DEVELOPMENT

Two classical models have been proposed for the development and progression of CRC from colon epithelial cells. The first model describes the process of transformation of adenoma to cancer, mainly initiated by mutations in the adenomatous polyposis coli (*APC*) gene. In this model, *APC* mutation is followed by mutations in Kirsten rat sarcoma viral oncogene homolog (*KRAS*)/neuroblastoma rat sarcoma viral oncogenes, mothers against decapentaplegic homolog 4, and finally, tumor protein p53 (*TP53*).

The other model describes the transformation from serrated polyps to cancer, with the driver mutation of catenin beta 1 secondary to mutations of *KRAS*/B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha[6]. These mutations eventually result in transforming growth factor-beta receptor type 2 (TGFRB2) overexpression[6]. The main signaling molecules involved are Wnt, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and TP53, which are hyperactivated[7]. Several other signaling pathways, involving hedgehog, erb-b2 receptor tyrosine kinase, ras homolog family member A, Notch, bone morphogenetic protein, Hippo, AMP-activated protein kinase, nuclear factor kB, and Jun N terminal kinase, also participate in the occurrence and development of CRC[8].

TRUNCATED *APC* MUTATION

Several studies have indicated that *APC* mutations are extremely important in both the mutation

frequency and different stages of CRC tumor development[9-11]. APC is the "gatekeeper" gene of the colorectal mucosal epithelium and the key molecule regulating colon epithelial cell homeostasis, polarity, and movement. *APC* acts as a tumor suppressor gene of CRC and 80% of sporadic CRCs harbor mutations, which are widely considered an early event in colorectal malignancy. Somatic point mutations of *APC* mainly occur in the mutation cluster region (MCR); however, *APC* contains several other mutations in its protein-coding region[9,10].

The most important cytological events after mutation in the MCR are the structural truncation of APC (amino acid sequence from 1362 to 1540, namely, the APC-2,3 repeats) and lack of axin, catenin, microtubules, and other binding sites[11]. Mutations in truncated APC result in the loss of the microtubule-binding properties of APC and further reduce microtubule stability. Truncating mutations cause *APC* to lose the properties of normal tumor suppressor genes and show "acquired" proto-oncogene properties, including abnormal cell proliferation and survival, chromosomal instability, and increased migration.

Truncated APC binds to and activates APC-stimulated guanine nucleotide exchange factor (ASEF), which is closely related to actin remodeling and movement and causes significant changes in cell structure and function[12]. Knockdown of *ASEF* or *APC*-truncating mutations markedly reduces cell migration; however, overexpression of full-length APC does not increase ASEF-mediated cell migration. The Golgi complex is a dynamic organelle that is essential for sorting, processing, and transporting proteins by which the stability of cellular structures is maintained.

Fragmentation of the Golgi apparatus is observed in age-related diseases including Alzheimer's disease, Parkinson's disease, and cancer[13]. The Golgi apparatus may be closely involved in the development of human diseases; however, the mechanisms and significance of its fragmentation are poorly understood. *APC*-truncating mutations induce fragmentation of the Golgi apparatus and lead to structural reorganization of cytoskeletal proteins and actin[13]. This causes cells to exhibit abnormal biological behavior, such as loss of polarity and increased migration[13]. Therefore, *APC*-truncating mutations driven by Golgi fragmentation are an important event in normal cells.

Mutation-rich regions of *APC* in the normal mucosa, LST, colorectal adenocarcinoma, and colorectal adenoma specimens were detected using polymerase chain reaction-single-strand conformation polymorphism[14]. The results of that study showed that while no *APC* mutations were observed in the normal mucosa of the large intestine, the mutation rates were 25%, 30%, and 27.8% in LST, colorectal adenocarcinoma, and colorectal adenoma, respectively[14]. The difference in rates between the specimens was not statistically significant and the results were consistent with other reported *APC* mutation rates of 15.5%-42.4% in LST specimens. Two recent studies showed *APC* mutation frequencies of 80% (10 samples) and 57% (14 samples) in LST[15]. Therefore, *APC* mutation may act as an important initiator in LST development[16].

TP53 AND *CRC*

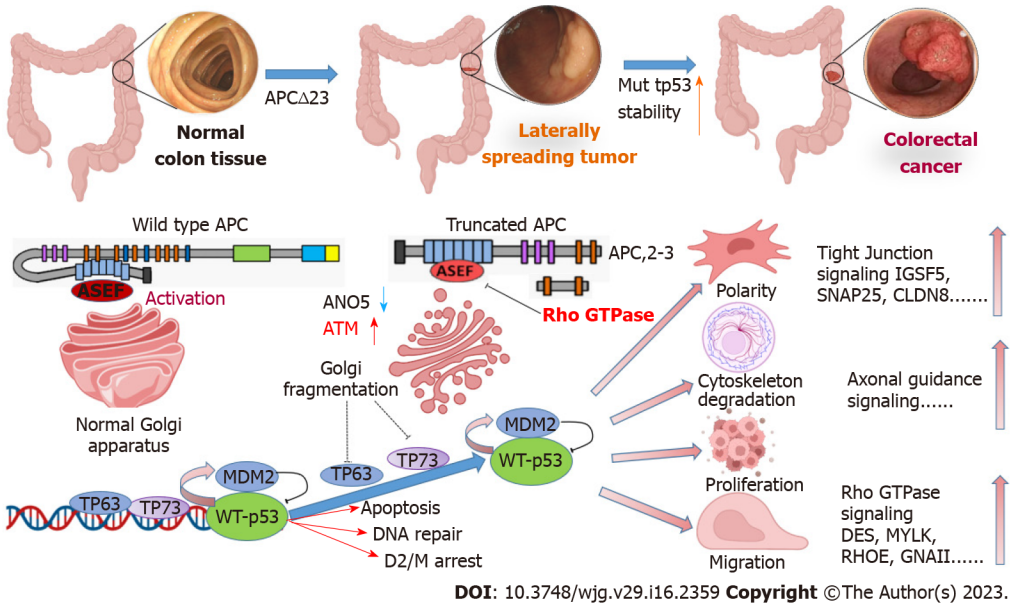
TP53 and *CRC* are important intracellular tumor suppressor genes, and the main biological function of *TP53* is the repair of cellular damage. Normal *TP53* can be used to monitor the integrity of genomic DNA in real time. During DNA damage, TP53 stops cell division at the G1/S phase to allow cells to have enough time to repair the damage. For irreparable DNA damage, TP53 induces programmed apoptotic cell death, thereby inhibiting the generation of possible mutant cancerous cells[17,18].

TP53 mutations primarily occur during the middle and late stages of carcinogenesis. Numerous *TP53* mutations reduce the proportion of wild-type *TP53* and weaken its function in monitoring genomic DNA integrity, thereby allowing tumorigenesis[19]. Studies on *TP53* and *LST* are scarce, and a recent comprehensive and unbiased screening of the genome, epigenome, and transcriptome was conducted based on the Cancer Genome Atlas (TCGA) database[14].

Bioinformatic data integrated from 11 LST samples and validated in an additional cohort of 84 benign colorectal injury samples, identified several high-frequency genetic, epigenetic, and transcriptional alterations[14]. Deletions occurred in chromosomes 1p, 5q, 14q, and 18, whereas doubling occurred in chromosomes 7, 8, 13, 19, and 20. Furthermore, these alterations were highly prevalent in the panel of the colorectal and rectal adenocarcinoma validation groups. The main signaling pathways associated with LST are axonal guidance, thyroid cancer, human embryonic stem cell pluripotency (Nanog homeobox), and Wnt/β-catenin.

Cohort validation studies compared 10 LSTs with 212 CRC samples with a focus on five major altered signaling pathways, Wnt, TGF β, PI3K, MAPK, and P53[20,21]. The results showed that the differences in the TGFβ and TP53 signaling pathways were significant[20,21]. The results suggested that ataxia-telangiectasia mutated (ATM), a very important molecule in the TP53 pathway, was significantly increased, which stabilized TP53 molecules. The expression of another very important gene, anoctamin 5 (*ANO5*), was significantly reduced, leading to mitochondrial fragmentation.

Our results based on TCGA data analysis also confirmed a significantly low expression level of *ANO5* in LST. The expression of the Golgi fragmentation-related genes *ATM* and *ANO5* was significantly different. This differential expression weakened the expression of the wild-type TP53 transcription



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Figure 1 Schematic diagram of mechanism by which adenomatous polyposis coli-truncating mutations regulate P53 to promote laterally spreading tumor transition to colorectal cancer. APC: Adenomatous polyposis coli; ASEF: Adenomatous polyposis coli-stimulated guanine nucleotide exchange factor; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein; IGSF5: Immunoglobulin superfamily member 5; SNAP25: Synaptosome associated protein 25; DES: Desmin; MYLK: Myosin light chain kinase; RHOE: Ras homolog gene family, member E; GNALL: G protein subunit alpha i1.

factors MDM2 proto-oncogene (MDM2) and TP73, and subsequently modulated TP53 stability in the TP53 signaling pathway. These two important signaling molecules are closely related to the stability of TP53 mutants. These data suggest that TP53 mutation has a unique molecular mechanism that differs from that in polyps and adenomas in CRC development from LST.

In our opinion, the progression of LST to CRC is distinct from the development of common polyps or adenoma carcinogenesis. Therefore, we proposed a two-stage cascade mutation hypothesis from LST to CRC (Figure 1): The driver stage and cancerous stage. In the driver stage, APC-truncating mutations drive Golgi fragmentation, resulting in reorganization of cellular structural proteins, thereby leading to abnormal polarity and lateral growth. In the cancerous stage, Golgi fragmentation further affects the repair mechanism of TP53 base mismatch mediated by the heat shock proteins MDM2, TP63, and TP73, resulting in increased stability of mutant TP53 and promotion of LST progression to carcinoma.

SPECIAL GENE EXPRESSION ANALYSIS

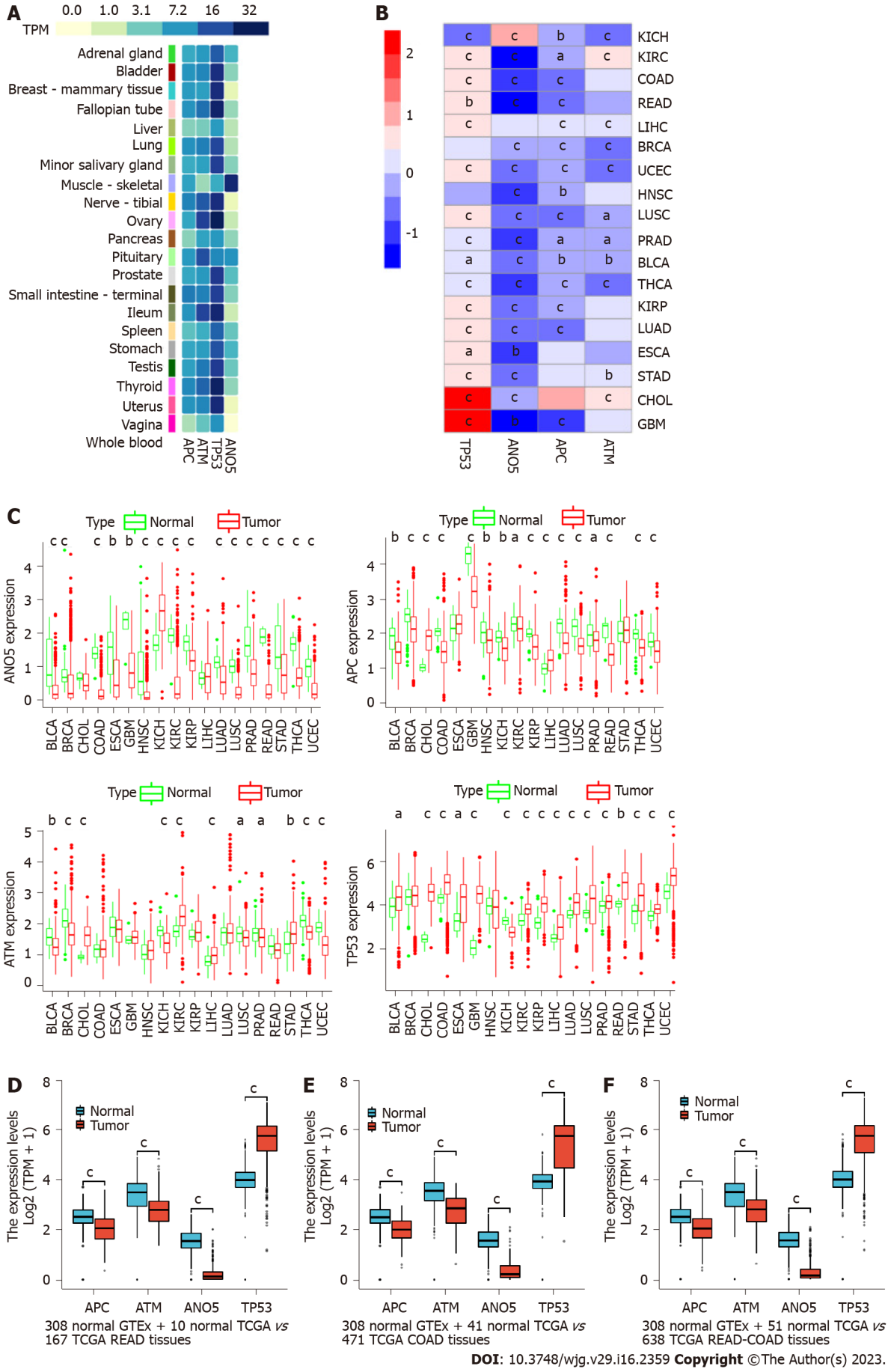
Based on the mRNA data of 25711 samples from 980 healthy donors in the genotype-tissue expression (GTEx) v8 database, pan-cancer mRNA-sequencing (mRNA-seq, n = 11057) and survival (n = 10121) data were downloaded from the TCGA database. We then performed a differential expression analysis of *ATM*, *ANO5*, *APC*, and *TP53* in pan-cancer and adjacent normal samples. Survival analysis of these genes was also performed in pan-cancers.

We analyzed the expression profiles of *ATM*, *ANO5*, *APC*, and *TP53* in human normal and pan-cancer samples and found that *ATM*, *ANO5*, and *APC* were significantly downregulated in rectal adenocarcinoma (READ), colon adenocarcinoma (COAD), and READ-COAD samples. In contrast, TP53 showed an obviously higher level in READ, COAD, and READ-COAD samples than in normal samples.

Furthermore, the survival analysis results indicated that *ATM*, *ANO5*, *APC*, and *TP53* expression were correlated with the overall survival (OS) of lung squamous cell carcinoma (LUSC), thyroid carcinoma (THCA), mesothelioma (MESO), pancreatic adenocarcinoma (PAAD), COAD, brain lower grade glioma (LGG), and breast invasive carcinoma (BRCA) patients.

Analysis of ATM, ANO5, APC, and TP53 expression in normal and pan-cancer samples

We analyzed the expression profiles of *ATM*, *ANO5*, *APC*, and *TP53* in normal human tissues based on transcripts per million (TPM) values from the GTEx v8 database. The results indicated that *ATM*, *ANO5*, *APC*, and *TP53* were highly or moderately expressed in most organs or tissues (1 < average TPM < 32), except that *ANO5* expression was low in the whole blood, vagina, and breast (average TPM < 1, Figure 2A).



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Figure 2 Analysis of ataxia-telangiectasia mutated gene, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 expression in

normal and pan-cancer samples. A: Expression profiles of ataxia-telangiectasia mutated (ATM), anoctamin 5 (ANO5), adenomatous polyposis coli (APC), and tumor protein 53 (TP53) in normal human organs or tissues; B: Heatmap of log2[fragments per kilobase of exon per million mapped fragments (FPKM) + 1] expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples. Red and blue represent upregulation and downregulation of gene expression, respectively; C: Log2(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples; D: Rectal adenocarcinoma (READ) and normal samples; E: Colon adenocarcinoma (COAD) and normal samples; F: READ-COAD and normal samples. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; COAD: Colon adenocarcinoma; READ: Rectal adenocarcinoma; LIHC: Liver hepatocellular carcinoma; BRCA: Breast invasive carcinoma; UCEC: Uterine corpus endometrial carcinoma; HNSC: Head and neck squamous cell carcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; BLCA: Breast invasive carcinoma; THCA: Thyroid carcinoma; KIRP: Kidney renal papillary cell carcinoma; LUAD: Lung adenocarcinoma; ESCA: Esophageal carcinoma; STAD: Stomach adenocarcinoma; CHOL: Cholangiocarcinoma; GBM: Glioblatoma; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

Then, we performed a differential expression analysis of mRNAs for these genes across 18 cancer types that had over five pairs of cancer-adjacent samples based on the $\log_2$[fragments per kilobase of exon per million mapped fragments (FPKM) + 1] data from TCGA. Figure 2B shows the heatmap of $\log_2$(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples where red and blue represent upregulation and downregulation, respectively]. Figure 2C shows the $\log_2$(FPKM + 1) expression status of *ATM, ANO5, APC*, and *TP53* between cancer and cancer-adjacent samples.

Table 1 shows that the expression of *TP53* was significantly upregulated in bladder cancer (BLCA), cholangiocarcinoma (CHOL), COAD, esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), LUSC, prostate adenocarcinoma (PRAD), READ, stomach adenocarcinoma (STAD), THCA, and uterine corpus endometrial carcinoma (UCEC) and downregulated in kidney chromophobe (KICH), compared with the expression levels in cancer-adjacent samples ($|\log_2 FC| > 0.19$, 4.99E-12 < P < 0.03). The *ANO5* level was significantly higher in KICH and obviously lower in BLCA, BRCA, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LUAD, LUSC, PRAD, READ, STAD, THCA, and UCEC than in cancer-adjacent samples ($|\log_2 FC| > 0.46$, 2.36E-26 < P < 0.003).

APC expression was significantly upregulated in CHOL and LIHC relative to that in cancer-adjacent samples and an obvious downregulation was noticed in BLCA, BRCA, COAD, GBM, HNSC, KICH, KIRC, KIRP, LUAD, LUSC, PRAD, READ, THCA, and UCEC ($|\log_2 FC| > 0.25$, 1.46E-15 < P < 0.03). In addition, the *ATM* expression was obviously upregulated in CHOL, KIRC, LIHC, and STAD compared to that in cancer-adjacent samples. In contrast, BLCA, BRCA, KICH, LUSC, PRAD, THCA, and UCEC ($|\log_2 FC| > 0.12$, 2.14E-16 < P < 0.049) were obviously downregulated.

We also analyzed the difference in expression among COAD, READ, and their cancer-adjacent samples based on the merged and batch-normalized TPM expression data of the GTEx and TCGA. The results showed that *ATM, ANO5*, and *APC* were significantly downregulated, whereas TP53 showed an obviously higher expression in READ (Figure 2D), COAD (Figure 2E), and READ-COAD (Figure 2F) samples than in normal samples (P < 0.001).

Association of ATM, ANO5, APC, and TP53 with cancer prognosis

To explore the role of *ATM, ANO5, APC*, and *TP53* in pan-cancer prognosis, we conducted a survival analysis in pan-cancers based on the $\log_2$(FPKM + 1) data and clinical survival data of 33 cancer types. The survival map of *ATM, ANO5, APC*, and *TP53* expression in pan-cancers indicated that the expression of these four genes was correlated with OS in LUSC, THCA, MESO, PAAD, COAD, LGG, and BRCA (Figure 3A). The relationship between *ATM, ANO5, APC*, and *TP53* and the OS of cancer patients ($^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$) was identified.

Briefly, LUSC or THCA patients with *ANO5* expression had a poor OS [1.40 < hazard ratio (HR) < 3.80, P = 0.014], whereas MESO or PAAD patients with *ANO5* expression showed a good OS (0.53 < HR < 0.57, 0.0031 < P < 0.018). *ATM* expression was indicative of a poor OS in COAD (HR = 1.70, P = 0.038). *TP53* expression was positively associated with a poor OS in BRCA (HR = 1.40, P = 0.038), LGG (HR = 1.60, P = 0.0067), and PAAD (HR = 12, P = 0.0033). In contrast, *ATM* expression exhibited a positive association with good OS in COAD (HR = 0.54, P = 0.012). Moreover, *APC* expression showed a good co-relation with OS in BRCA (HR = 0.49, P = 9E-06, Figure 3B).

CONCLUSION

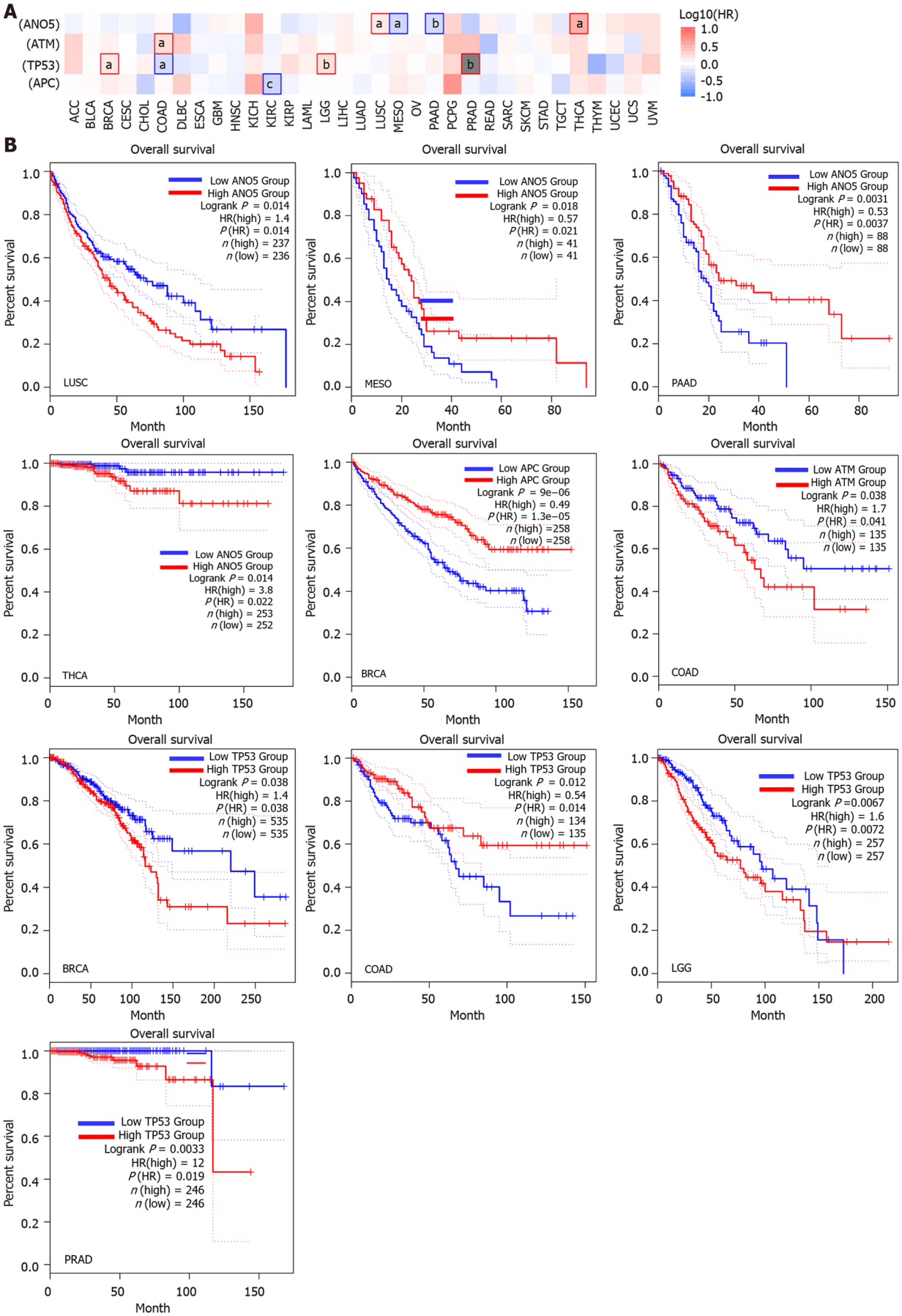
APC-truncating mutations driven by Golgi fragmentation are a very important cellular event, which can cause loss of the microtubule binding properties of APC. This effect further reduces microtubule stability, resulting in abnormal cell proliferation and survival, chromosomal instability, and increased migration. Downstream characteristics of Golgi fragmentation include gene expression alterations with *ATM* upregulation and *ANO5* downregulation, which are significant in LST. These observations suggest

Table 1 Identification of ataxia-telangiectasia mutated, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 expression in pan-cancer samples

Cancer type	APC		TP53		ATM		ANO5	
	LogFC	*P* value	LogFC	*P* value	LogFC	*P* value	LogFC	*P* value
BLCA	-0.28889	0.005445	0.314908	0.032102	-0.27112	0.002207	-0.75984	5.46E-07
BRCA	-0.37268	1.46E-15	0.070558	0.261271	-0.41783	2.30E-16	-0.26734	2.03E-17
CHOL	0.798977	4.07E-06	2.043449	1.51E-07	0.718097	8.17E-06	-0.08998	0.123426
COAD	-0.57688	2.42E-15	0.505369	4.40E-09	0.086761	0.429393	-1.16109	3.06E-24
ESCA	0.134442	0.480134	0.792589	0.011648	-0.0696	0.629714	-0.81744	0.003288
GBM	-1.01588	0.000957	2.388451	0.000173	0.016551	0.761476	-1.26367	0.001745
HNSC	-0.18476	0.004628	-0.08774	0.958273	0.116251	0.114312	-0.82404	7.30E-09
KICH	-0.32313	0.003139	-0.63636	1.15E-07	-0.42688	1.83E-05	0.889727	7.05E-07
KIRC	-0.12313	0.033897	0.452895	2.19E-16	0.476935	2.14E-16	-1.43405	4.66E-34
KIRP	-0.34452	1.85E-05	0.784867	3.85E-12	0.110608	0.113455	-0.50628	2.70E-09
LIHC	0.253395	8.34E-06	0.41186	5.80E-06	0.187905	0.000776	0.070468	0.537737
LUAD	-0.4475	1.24E-13	0.430958	3.79E-09	0.14328	0.135749	-0.46868	9.13E-13
LUSC	-0.51847	1.39E-15	0.467462	0.000189	-0.14078	0.016441	-0.73565	5.71E-25
PRAD	-0.17991	0.011276	0.193039	0.000387	-0.12553	0.049659	-0.89276	6.11E-19
READ	-0.71729	3.89E-05	0.638392	0.007547	-0.18753	0.144797	-1.5861	1.99E-07
STAD	0.025202	0.628002	0.660337	1.23E-06	0.321073	0.001535	-0.63734	2.77E-05
THCA	-0.36112	1.91E-13	0.320993	4.99E-12	-0.40106	1.15E-13	-0.89401	2.36E-26
UCEC	-0.34584	3.78E-06	0.552104	1.66E-08	-0.56028	9.35E-14	-0.63913	2.84E-15

BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; ESCA: Esophageal carcinoma; GBM: Glioblatoma; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma; READ: Rectal adenocarcinoma esophageal; STAD: Stomach adenocarcinoma; THCA: Thyroid carcinoma; UCEC: Uterine corpus endometrial carcinoma; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

the existence of a unique and novel pathway for the development of CRC from LST, and future studies of potential CRC treatment should focus on this newly identified mechanism.



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Figure 3 Association of ataxia-telangiectasia mutated gene, anoctamin 5, adenomatous polyposis coli, and tumor protein 53 with

prognosis of cancer in patients. A: Survival map of ataxia-telangiectasia mutated (*ATM*) gene, anoctamin 5 (*ANO5*), adenomatous polyposis coli (*APC*), and tumor protein 53 (*TP53*) expression in pan-cancers; B: Relationship of *ATM, ANO5, APC*, and *TP53* expression with overall survival of cancer patients. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001. ACC: Adrenocortical carcinoma; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblatoma; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute myeloid leukemia; LGG: Brain lower grade glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectal adenocarcinoma esophageal; SARC: Sarcomav; SKCM: Skin cutaneous melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular germ cell tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine corpus endometrial carcinoma; UCS: Uterine carcinosarcoma; UVM: Uveal melanoma; HR: Hazard ratio; APC: Adenomatous polyposis coli; ANO5: Anoctamin 5; ATM: Ataxia-telangiectasia mutation; TP: Tumor protein.

FOOTNOTES

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OPINION REVIEW

Current status and progress in laparoscopic surgery for gallbladder carcinoma

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Abstract

Gallbladder carcinoma (GBC) is the most common biliary tract malignancy associated with a concealed onset, high invasiveness and poor prognosis. Radical surgery remains the only curative treatment for GBC, and the optimal extent of surgery depends on the tumor stage. Radical resection can be achieved by simple cholecystectomy for Tis and T1a GBC. However, whether simple cholecystectomy or extended cholecystectomy, including regional lymph node dissection and hepatectomy, is the standard surgical extent for T1b GBC remains controversial. Extended cholecystectomy should be performed for T2 and some T3 GBC without distant metastasis. Secondary radical surgery is essential for incidental gallbladder cancer diagnosed after cholecystectomy. For locally advanced GBC, hepatopancreatoduodenectomy may achieve R0 resection and improve long-term survival outcomes, but the extremely high risk of the surgery limits its implementation. Laparoscopic surgery has been widely used in the treatment of gastrointestinal malignancies. GBC was once regarded as a contraindication of laparoscopic surgery. However, with improvements in surgical instruments and skills, studies have shown that laparoscopic surgery will not result in a poorer prognosis for selected patients with GBC compared with open surgery. Moreover, laparoscopic surgery is associated with enhanced recovery after surgery since it is minimally invasive.

Key Words: Gallbladder carcinoma; Laparoscopic surgery; Simple cholecystectomy; Extended cholecystectomy; Hepatopancreatoduodenectomy; Incidental gallbladder cancer

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Core Tip: Gallbladder carcinoma (GBC) is the most common biliary tract malignancy with a poor prognosis. Radical surgery is the mainstay of treatment, and the surgical extent depends on the tumor stage. Meanwhile, laparoscopic surgery has the advantage of enhanced recovery after surgery because it is minimally invasive, and has been widely used to treat gastrointestinal malignancies. Although GBC was once regarded as a contraindication for laparoscopic surgery, with improved surgical instruments and skills, recent studies have shown that laparoscopic surgery will not lead to a poorer prognosis compared with open surgery among selected patients with GBC in specialized centers.

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INTRODUCTION

Gallbladder carcinoma (GBC) has the highest incidence among malignant tumors of the biliary system, accounting for 80%-95% of all biliary tract cancers. GBC is more common in Chile, Japan and northern India[1]. Although GBC has a relatively low incidence of about 1.2% of all malignant tumors of the digestive system[2,3], its invasiveness is extremely high. The median survival time is six months, and the 5-year survival rate is less than 5%[4,5]. The prognosis is closely related to the tumor stage[5]. The high degree of malignancy of GBC is mainly due to the lack of submucosa and the relatively thin muscular layer of the gallbladder, and tumor cells are more likely to invade surrounding tissues and organs like the liver quickly[6]. Because of the frequent absence of typical symptoms, over 1/3 of patients are diagnosed with advanced GBC without the opportunity of radical operation. Only 15%-47% of patients with GBC diagnosed preoperatively will meet the indication for surgical resection. However, radical surgery remains the cornerstone of treatment because the effect of adjuvant therapy for GBC is very limited, and the surgical approach depends on the tumor stage (Table 1)[3]. With improvements in diagnostic and surgical techniques, the prognosis of patients with GBC who underwent radical surgery has been significantly improved in recent years[7].

Laparoscopic surgery has been widely used with the advent of "Enhanced Recovery After Surgery (ERAS)". With the advantages such as reducing the incision and magnifying the view, this surgical method can reduce intraoperative bleeding, alleviate postoperative pain, promote earlier oral intake, reduce complications like wound infection, and shorten the duration of hospitalization, achieving the goal of ERAS[8]. Since the rise of laparoscopic cholecystectomy in the early 1990s[9], laparoscopic surgery has been widely used to treat typical gastrointestinal malignant tumors. However, GBC was once regarded as a contraindication for laparoscopic surgery for the following main reasons[10,11]: (1) Bile spillage associated with intraoperative gallbladder perforation and repeated manipulation through the trocars could increase the incidence of peritoneal dissemination or port site metastasis (PSM); (2) The oncologic adequacy and safety of laparoscopic radical surgery for GBC still need to be verified by high-quality prospective studies; and (3) There were technical difficulties related to the procedure, such as lymph node dissection of hepatoduodenal ligament and around the hepatic artery, liver resection and bile duct resection in laparoscopic approaches. However, with the improvement of preoperative diagnosis of GBC, the progress of surgical skills and laparoscopic equipment, and the avoidance of bile spillage by careful manipulation and extensive implementation of retrieval bags, the incidence of peritoneal dissemination or PSM associated with laparoscopic surgery for GBC has been significantly reduced, with no significant difference in survival outcomes compared with open surgery in recent literature[10]. According to a systematic review by Berger-Richardson *et al*[12], the incidence of PSM after laparoscopic cholecystectomy for incidental GBC (IGBC) in the historic era (1991-1999) was about 18.6% and decreased to 10.3% in the modern era (2000-2014). Since the incidence of incision recurrence after open cholecystectomy is approximately 7%[12], the gap between the two approaches is gradually narrowing. Several studies have shown that there is no difference in the number of harvested lymph nodes by laparoscopy or laparotomy in radical resection of rectal cancer[13]. In addition, laparoscopy has been widely used in hepatectomy, which shows that the feasibility and safety of laparoscopic lymph node dissection and hepatectomy have been gradually proved by surgical experts[14]. Moreover, the development of laparoscopic suturing skills makes laparoscopic bile duct reconstruction possible[15]. The surgical extent of GBC varies greatly in different tumor stages. In order to ensure the safety and oncological adequacy of resection, surgeons should strictly select patients undergoing laparoscopic surgery for GBC[16]. This review will discuss the application of laparoscopic surgery in GBC according to the surgical approach and whether the cancer is IGBC.

Table 1 Summary of gallbladder carcinoma T staging according to the American Joint Committee on Cancer 8th edition and the corresponding surgical approach

	AJCC 8th T staging classification	Surgical approach
Tis	Carcinoma *in situ*	Simple cholecystectomy
T1a	Tumor invades the lamina propria	Simple cholecystectomy
T1b	Tumor invades the muscular layer	Extended cholecystectomy including cholecystectomy + lymphadenectomy ± hepatectomy (current consensus)/simple cholecystectomy (under debate)
T2a	Tumor invades the perimuscular connective tissue on the peritoneal side, without involvement of the serosa	Extended cholecystectomy including cholecystectomy + lymphadenectomy ± hepatectomy ± bile duct resection and reconstruction
T2b	Tumor invades the perimuscular connective tissue on the hepatic side, with no extension into the liver	Extended cholecystectomy including cholecystectomy + lymphadenectomy + hepatectomy ± bile duct resection and reconstruction
T3	Tumor perforates the serosa (visceral peritoneum) and/or directly invades the liver and/or one other adjacent organ or structure, such as the stomach, duodenum, colon, pancreas, omentum or extrahepatic bile ducts	Extended cholecystectomy including cholecystectomy + lymphadenectomy + hepatectomy ± bile duct resection and reconstruction (some T3 without distant metastasis)/hepatopancreatoduodenectomy (under debate)
T4	Tumor invades the main portal vein or hepatic artery or invades two or more extrahepatic organs or structures	No significant benefit from surgery

AJCC: American Joint Committee on Cancer.

SIMPLE CHOLECYSTECTOMY

For Tis and T1a GBC, R0 resection can be achieved through simple cholecystectomy, which meets the standard of oncological safety, and both laparoscopic and open surgery can reach the postoperative overall survival rate of 95%-100%[10]. However, it is controversial whether for T1b GBC, simple cholecystectomy or extended cholecystectomy, which includes lymph node dissection and hepatectomy, is oncologically safe[17], although the latter is recommended by the current guidelines[17]. A study of 536 T1b GBC subjects from Surveillance, Epidemiology, and End Results database showed that extended cholecystectomy could improve disease-specific and overall survival of those patients[18]. In addition, Butte *et al*[19] found that 35% of patients with T1b GBC had residual disease after simple cholecystectomy, which supports the necessity of extended cholecystectomy for patients with T1b GBC.

However, more studies in recent years have shown that simple cholecystectomy does not adversely affect the long-term prognosis of patients with T1b GBC[20-22], and the choice of surgical extent mainly depends on the experience of the surgeon[23]. In a meta-analysis in 2017, including 22 publications published in MEDLINE since 1994, Lee *et al*[17] compared the relationship between surgical extents *via* laparoscopic or open surgery and the prognosis of T1 GBC among patients with T1b GBC. They found that the risk difference between simple cholecystectomy and extended cholecystectomy was 0.03, while the risk ratio was 1.06, indicating no significant difference in overall survival outcomes between the two surgical extents. Recent studies have reported that long-term outcomes of patients with early GBC after laparoscopic cholecystectomy, which is now widely adopted, are comparable to laparotomy[24,25]. Therefore, the laparoscopic approach is safe and feasible for patients with early GBC undergoing simple cholecystectomy, and its minimally invasive characteristics can accelerate the postoperative rehabilitation process[26,27]. However, more large cohort studies are needed to confirm the long-term prognosis of this approach, given the low diagnostic rate and staging accuracy of T1 GBC. It should be taken into special consideration that gallbladder perforation caused by forceps during the operation will cause tumor dissemination. For suspected GBC, the resected gallbladder should be removed completely and extracted with a retrieval bag during laparoscopic cholecystectomy to avoid tumor dissemination caused by bile spillage into the abdominal cavity or port sites[24].

EXTENDED CHOLECYSTECTOMY

Extended cholecystectomy for GBC is now mainly recommended for T1b, T2 and some T3 patients without distant metastasis, which includes cholecystectomy + regional lymph node dissection ± adjacent hepatectomy ± bile duct resection and reconstruction[16]. A number of studies have shown that it is safe and effective to perform laparoscopic extended cholecystectomy for patients who meet criteria such as no surgical contraindications, no severe abdominal adhesion and tolerance of pneumoperitoneum. The postoperative recurrence and survival outcomes are comparable to and even better than those of laparotomy[28,29]. According to the single-center retrospective study of patients with T2 GBC treated from 2004 to 2017 by Jang *et al*[11], there was no significant difference between laparoscopic and open

extended cholecystectomy in terms of the number of retrieved lymph nodes and 5-year survival rate, and postoperative hospital stay was significantly shorter in the laparoscopic group. A recent meta-analysis including 14 studies comparing laparoscopic and open extended cholecystectomy for GBC published in several databases up to April 6, 2021, found that laparoscopic surgery had a lower risk of death than open surgery for T3 GBC, while there was no significant difference in death between the two methods for T1 and T2 GBC. In addition, the survival rate after laparoscopic surgery was higher than that after open surgery for the first two years for T2 and T3 GBC, but the three-year and five-year survival rates were similar between the two groups regardless of the tumor stage. Lastly, no significant difference in the overall recurrence was found between the two surgical approaches. The above results also confirm the feasibility and safety of laparoscopic extended cholecystectomy[30].

Lymph node dissection

Lymph node dissection during extended cholecystectomy for GBC is mainly used to stage the patient [31], but the optimal extent of regional lymphadenectomy is still under debate. In the published studies, the extent of lymph node dissection for GBC mainly includes lymph nodes around the hepatoduodenal ligament, and some centers also emphasize the necessity to dissect lymph nodes in the posterior superior pancreaticoduodenal area and along the common hepatic artery at the same time because of the high frequency of metastasis in this area and the possibly improved survival rate after complete resection[27,32,33]. More extensive dissection of the aorto-caval, celiac and superior mesenteric artery nodes has limited effect and insignificant survival benefit. However, some centers advocate aorto-caval node sampling at the beginning of the operation to estimate the presence of distant metastasis. Patients with positive lymph node biopsies in this area can hardly benefit from extended cholecystectomy for GBC[34]. Recent studies have shown that in selected patients with GBC, the extent of laparoscopic lymph node dissection and the number of retrieved lymph nodes are similar to open surgery with few intraoperative and postoperative complications[15,28,29,35,36]. A prospective cohort study by Yoon *et al* [36] showed that the median number of harvested lymph nodes was seven in the 32 patients with T1b-T2 GBC who underwent laparoscopic extended cholecystectomy in their center from 2004 to 2014, exceeding the minimum number of six recommended by the 8th AJCC[21,37], and there was no local recurrence within the extent of lymphadenectomy within 10 years after surgery[36], indicating the feasibility and oncological adequacy of laparoscopic lymph node dissection in patients with GBC. Vega *et al*[34] compared laparoscopic extended cholecystectomy with open surgery in 35 cases of GBC treated in their center, and found that the median number of lymph nodes harvested by the two methods was both six, and there was no significant difference in residual disease, recurrence rate, postoperative complications and 90-d mortality between the two surgical approaches.

Liver resection

Negative margins should be achieved in hepatectomy for patients with GBC in order to reduce tumor recurrence caused by liver micrometastasis. If the gallbladder is severely adherent to the liver, the attached thin layer of liver tissue is often removed in conjunction with the gallbladder to avoid bile spillage caused by gallbladder damage. The most common surgical extent is wedge resection for at least 2 cm of the liver bed, and IVb/V resection is also performed in some centers[16,34]. For some patients with T3 GBC, (extended) right hepatectomy can be performed to achieve R0 resection according to the patient's tolerance, but its clinical benefits need to be further confirmed as extensive hepatectomy will increase the rate of postoperative complications such as liver failure[1]. Current studies have reported the feasibility and safety of laparoscopic wedge resection or IVb/V resection[28,35,38], but there is no consensus on the best extent of hepatectomy, and no clinical data have confirmed the theoretical advantage of IVb/V resection over wedge resection[16]. A multicenter retrospective study by Lee *et al* [39] found that there was no significant difference in the 5-year survival rate or recurrence-free survival rate after wedge resection or IVb/V resection of the liver in patients with T2 GBC who underwent extended cholecystectomy, and some other researches also reported similar results[40,41].

It is worth noting that the hepatic-side and peritoneal-side GBC may have different prognoses. According to a multicenter retrospective study of patients with T2 GBC, the rates of nodal involvement, liver metastasis, postoperative intrahepatic recurrence and vascular and nerve invasion were higher in hepatic-side GBC, and the prognosis was worse than that of peritoneal-side GBC; however, there were no such differences in those with T1 and T3 GBC[42]. Some studies reported that the density of large vessels increased significantly in the deep layer of the gallbladder wall. The hepatic side of the gallbladder was drained by short veins directly connected to the intrahepatic portal veins, with the peritoneal side drained by 1 or 2 cystic veins terminating in the hepatic parenchyma or at the hepatic hilum[43]. The retrospective study suggested that the density of vessels and length of the drainage path caused the difference in the incidence of hepatic, vascular and lymphatic metastasis between hepatic-side and peritoneal-side GBC[42]. Another multicenter retrospective study showed that for patients with hepatic-side T2 GBC, the 5-year survival rate was higher in patients who underwent extended cholecystectomy, including both regional lymphadenectomy and hepatectomy, than in patients who underwent extended cholecystectomy without hepatectomy. In addition, the extent of hepatectomy did not affect the prognosis. Furthermore, for patients with peritoneal-side T2 GBC who underwent lymph node dissection, the 5-year survival rate was not affected by hepatectomy or the extent of lymphaden-

ectomy. Therefore, it is considered that extended cholecystectomy, including lymphadenectomy and R0 hepatectomy, is essential for patients with hepatic-side T2 GBC, while patients with peritoneal-side T2 GBC can only undergo cholecystectomy and lymph node dissection without hepatectomy[39].

Bile duct resection and reconstruction

Indications for bile duct resection in patients with GBC include a positive cystic duct margin, direct tumor invasion of the bile duct and inflammation or scarring around the hepatoduodenal ligament that compromise lymphadenectomy[16]. It is not recommended to perform routine bile duct resection for patients with GBC because it increases the risk of complications without improving the survival rate[44-47]. The present literature has proved the feasibility of laparoscopic bile duct resection in patients with GBC[48]. With the accumulation of experience in laparoscopic surgery in choledochal cysts and pancreatoduodenectomy, the need for bile duct resection and reconstruction is no longer a contraindication of laparoscopic extended cholecystectomy for GBC[16].

HEPATOPANCREATODUODENECTOMY

The gallbladder is adjacent to the liver, duodenum and colon. For patients with locally advanced GBC, it is feasible to achieve R0 resection with hepatopancreatoduodenectomy (HPD) and improve the long-term survival rate. However, only about 10% of the patients can meet the conditions for HPD[1], which include: Tumor at the body or bottom of the gallbladder (hepatic bed type); tumor invading the hepatic hilum (hilar type); massive mass (hepatic bed + hilar type); extensive regional lymph node metastasis (lymph node type); tumor invading the distal bile duct or duodenum; and lymph node metastasis around the head of the pancreas. The contraindications include chronic hepatic diseases, severe comorbidities, R2 resection, paraaortic lymph node metastasis, peritoneal dissemination and distant metastasis. Postoperative mortality and the risk of complications such as liver failure, pancreatic fistula and biliary leakage are extremely high[1]. It is reported that the in-hospital morbidity rate after HPD is more than 10%[49]. Less than 1000 cases of this surgical approach have been reported in the past 50 years[49,50]. Dr Kasumi of Japan performed the first HPD for a patient with GBC invading the duodenum in 1974[51,52]. Takasaki *et al*[53] performed HPD on five patients with GBC invading the duodenum and pancreatic head in 1980. The 30-d mortality was 60%, and the survival time of the other two patients was five months and 16 mo, respectively[1,53]. However, with the improvement in surgical and anesthetic techniques and perioperative management, the prognosis of HPD has been improved. It is reported that the 3-year and 5-year survival rates after HPD are 48% and 37% respectively[54], and more surgeons choose to try this procedure because it has a better prognosis than unresectable tumors [55].

Because of the technical difficulty and high risks of laparoscopic HPD, only four cases of locally advanced GBC or extrahepatic cholangiocarcinoma have been reported to undergo this surgical approach so far (Table 2)[56-59]. Despite the postoperative complications such as bile leakage and delayed gastric emptying[56], the successful implementation of laparoscopic HPD in the four cases has proven its safety and feasibility. This surgery should be performed in large volume centers[1], and the surgeons should have sufficient experience in laparoscopic pancreaticoduodenectomy and laparoscopic hepatectomy[60,61]. Patients who only need a small extent of hepatectomy should be selected as far as possible to reduce postoperative complications. If the patients need major hepatectomy, portal vein embolization should be performed before the operation to increase the remnant volume and avoid postoperative liver failure[49,62]. For patients with obstructive jaundice and cholangitis, bile drainage should be performed preoperatively to improve hepatic function and promote postoperative remnant liver regeneration[63]. The risk of pancreatic fistula after pancreatoduodenectomy in patients with GBC is usually greater than that in patients with adenocarcinoma of the pancreatic head attributed to the soft texture of the pancreatic gland and small pancreatic duct, which could be reduced by two-stage pancreatojejunostomy, external drainage of pancreatic fluid and wrapping omental flap[64].

INCIDENTAL GALLBLADDER CANCER

According to the literature, the incidence of IGBC after laparoscopic cholecystectomy ranges from 0.19% to 3.3% and has increased significantly with the widespread use of laparoscopic cholecystectomy[65]. About 47%-70% of GBC cases are incidentally found during or after cholecystectomy[34], and 45%-60% of patients with IGBC have residual disease after the initial cholecystectomy[45,66,67]. Patients with IGBC are usually at the early stage, and reresection can significantly improve oncological outcomes for patients with T1b-T3 GBC without distant metastasis[68-70]. For patients with bile spillage, positive margin, poorly differentiated tumor or high risks of tumor dissemination during the initial cholecystectomy, it is recommended to perform laparoscopy before secondary radical cholecystectomy to detect metastases that are difficult to be found by preoperative imaging and avoid ineffective reresection[71,72]. Inflammatory adhesion and fibrosis around the hepatoduodenal ligament and the

Table 2 Summary of the published cases of laparoscopic hepatopancreatoduodenectomy for locally advanced gallbladder carcinoma or extrahepatic cholangiocarcinoma

Ref.	Country	Age (yr)	Diagnosis	Operation	Operation duration (min)	Main complication	Hospital stay (d)
Zhang *et al*[57], 2014	China	61	ECC invading the duodenum	LPD + LRH	600	Bile leakage	16
Chong and Choi [58], 2019	South Korea	73	ECC	LPD + LLH	510	Cystitis	16
James *et al*[59], 2021	India	73	GBC infiltrating the CBD	LPD + segments IVb and V	610	Delayed gastric emptying	12
Yao[56], 2022	China	75	ECC + GBC	LPD + segments IVb and V	380	No	12

ECC: Extrahepatic cholangiocarcinoma; GBC: Gallbladder carcinoma; CBD: Common bile duct; LPD: Laparoscopic pancreatoduodenectomy; LRH: Laparoscopic right hemihepatectomy; LLH: Laparoscopic left hemihepatectomy.

gallbladder bed significantly increase the difficulty of radical reoperation for IGBC. However, a few studies have reported the feasibility of laparoscopic radical reresection for IGBC[15,28,73-75] and shown prognoses comparable to that of laparotomy in selected early GBC[70,76]. However, the effect of laparoscopic reresection in patients with IGBC after cholecystectomy for acute cholecystitis needs to be further studied[77-79]. The specimen of the previous operation should be assessed again by a specialized pathologist before reoperation for T stage[19,80], the tumor location (hepatic-side or peritoneal-side), a positive bile duct margin[42], peritoneal and lymphovascular invasion and the presence of Rokitansky-Aschoff sinuses[81], which will increase the rate of conversion to open surgery[34]. Although there is a risk of PSM after cholecystectomy for IGBC, routine port site resection is not recommended because it can't improve the oncological outcomes or reduce recurrence attributed to the high rate of combined peritoneal metastasis, and it can increase the risk of morbidities like incisional hernia[82].

DISCUSSION

Laparoscopic surgery has many advantages over open surgery. Firstly, for benign diseases, which can't be completely excluded from GBC before operation, such as xanthogranulomatous cholecystitis, laparoscopic surgery can retain the opportunity of minimally invasive treatment after frozen section analysis of the specimen is confirmed. Secondly, laparoscopy can provide a clearer surgical field, and laparoscopic exploration can detect liver or peritoneal metastases that are difficult to detect preoperatively, reducing the incidence of unnecessary laparotomy[10]. Thirdly, laparoscopic surgery can reduce postoperative complications such as ileus and infection by reducing contact between the viscera and external environment[83,84]. Finally, minimally invasive surgery can not only accelerate rehabilitation by reducing the incision, alleviating pain, reducing blood loss and promoting early mobilization and oral intake but also initiate postoperative adjuvant therapy earlier[85], which could improve quality of life and prolong long-time survival of the patients[86].

Recent studies have proven the short-term benefits of laparoscopic surgery compared to laparotomy for GBC. A single-center retrospective study by Dou *et al*[87], including 99 patients with T2 and T3 stage GBC who underwent radical resection, showed that compared with open surgery, the laparoscopic group had lower intraoperative bleeding volume (233.91 ± 26.35 mL *vs* 461.25 ± 53.15 mL, $P < 0.01$) and shorter postoperative hospital stay (10.32 ± 0.60 d *vs* 14.74 ± 0.91 d, $P < 0.01$); although it had longer operation time (292.35 ± 14.41 min *vs* 249.02 ± 13.30 min, $P = 0.033$). Lymph node yield (9.39 ± 0.68 *vs* 8.26 ± 0.52, $P = 0.208$) and incidence of postoperative morbidities, including bile leakage (0.11 *vs* 0.07, $P = 0.521$), postoperative bleeding (0.05 *vs* 0.02, $P = 0.448$) and abdominal abscess (0.05 *vs* 0.07, $P = 0.738$) were similar between the two groups[87]. Another retrospective analysis of 102 patients with GBC reported that the patients who underwent laparoscopic surgery experienced a shorter postoperative activity time (2 ± 1 d *vs* 4 ± 1 d, $P < 0.001$), eating time (2 ± 1 d *vs* 4 ± 2 d, $P < 0.001$) and drainage tube removal time (4 ± 3 d *vs* 6 ± 3 d, $P < 0.001$) compared with those who underwent open surgery[88]. Similarly, according to the 18 studies comparing laparoscopic and open radical cholecystectomy for GBC analyzed by Lv *et al*[89], the laparoscopic group had a significantly smaller volume of intraoperative blood loss, a shorter time of drainage tube extraction and diet recovery, a lower rate of postoperative complications such as pulmonary infection and thrombus formation (which was 10.1% compared with 15.8%) and a shorter length of postoperative hospital stay. The shorter hospital stay is theoretically because of reduced wound-related pain, early-period ambulation and earlier gastrointestinal peristalsis. Operative time, intraoperative gallbladder violation, R0 resection rate,

lymph node yield and overall recurrence rate were comparable in the two groups[89]. Predictive factors for conversion to open surgery may include a positive liver margin, massive intraoperative bleeding and an interval between surgeries of more than 60 d, which may cause severe abdominal adhesions[34]. In the prospective study of Cho *et al*[24], including 33 patients with early-stage GBC who underwent laparoscopic surgery, three patients with liver invasion noted by diagnostic laparoscopy had their procedure converted to laparotomy, and another conversion occurred owing to bleeding during locoregional laparoscopic lymphadenectomy. A retrospective study showed that 7 out of 30 patients undergoing laparoscopic extended cholecystectomy with bi-segmentectomy in their center required conversion to open surgery due to distortion of anatomical landmarks and suspected involvement of extrahepatic organs that caused technical difficulty[90]. The rate of conversion to open surgery decreases with the improvement of surgical experience and equipment.

Moreover, laparoscopic surgery will not worsen the survival outcomes compared with open surgery in selected early-stage GBC by experienced surgeons *via* improved diagnosis rate, staging accuracy and precision of operation to avoid bile spillage. A study by Yoon *et al*[36] showed that among the 45 patients with GBC who underwent laparoscopic extended cholecystectomy in their center, the 5-year survival rate of T1a and T1b GBC was 100%, and that of T2 GBC was more than 90%. Only four patients experienced recurrence postoperatively, which were all distant metastases. Itano *et al*[29] compared 16 patients with T2 GBC who underwent laparoscopic extended cholecystectomy with 14 patients who underwent open surgery and found no significant difference in disease-free or overall survival rate between the two groups. However, anatomical features such as thin gallbladder walls and the presence of Rokitansky-Aschoff sinuses make it difficult to evaluate the depth of tumor invasion, and the preoperative staging accuracy is only 40%[91]. Endoscopic or laparoscopic ultrasonography is superior to traditional abdominal ultrasonography and computed tomography in diagnosing T staging[92]. Only a few surgeons have rich experience in laparoscopic radical surgery for GBC, and no consensus has been reached on this operation. Steps such as laparoscopic lymph node dissection, hepatectomy and choledochojejunostomy demand high requirements on surgical instruments and techniques. For patients with a massive mass, duodenal or colonic invasion, jaundice or hilar involvement, more surgeons still prefer open surgery[34]. In addition, regarding the higher cost of laparoscopic surgery from the use of consumable materials and the possibility of conversion to laparotomy, some experts and patients still have concerns and disputes about laparoscopic surgery for GBC[10]. This surgical approach is still in the early stage of the adoption curve. More multicenter prospective studies are needed to confirm the safety and efficacy of laparoscopic surgery for GBC[16].

CONCLUSION

For strictly selected patients with early GBC, long-term survival outcomes of laparoscopic surgery are comparable to that of open surgery, and laparoscopic surgery has the advantage of accelerating rehabilitation because of its minimally invasive characteristics. However, as the progress of minimally invasive treatment for GBC is relatively slow, more studies are needed to further confirm its oncological safety and efficacy and improve the standardization of the procedures of laparoscopic surgery for GBC.

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REVIEW

Molecular regulation mechanism of intestinal stem cells in mucosal injury and repair in ulcerative colitis

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Abstract

Ulcerative colitis (UC) is a chronic nonspecific inflammatory disease with complex causes. The main pathological changes were intestinal mucosal injury. Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5)-labeled small intestine stem cells (ISCs) were located at the bottom of the small intestine recess and inlaid among Paneth cells. LGR5+ small ISCs are active proliferative adult stem cells, and their self-renewal, proliferation and differentiation disorders are closely related to the occurrence of intestinal inflammatory diseases. The Notch signaling pathway and Wnt/β-catenin signaling pathway are important regulators of LGR5-positive ISCs and together maintain the function of LGR5-positive ISCs. More importantly, the surviving stem cells after intestinal mucosal injury accelerate division, restore the number of stem cells, multiply and differentiate into mature intestinal epithelial cells, and repair the damaged intestinal mucosa. Therefore, in-depth study of multiple pathways and transplantation of LGR5-positive ISCs may become a new target for the treatment of UC.

Key Words: Molecular regulation; Mucosal injury; Regeneration; Ulcerative colitis

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Core Tip: Intestinal mucosal injury is an important pathological change in ulcerative colitis (UC), and Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5)-positive intestinal stem cells play an important role in the repair of intestinal mucosal injury. Through in-depth study of multiple signals, LGR5-positive intestine stem cell transplantation therapy may become an important means to treat UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing inflammatory disease of the intestinal tract[1]. The course of the disease is prolonged and often brings heavy physiological, psychological and economic burdens to patients. Clinical remission based on symptom improvement does not alter the natural course of UC, and mucosal healing has been the primary therapeutic target of UC in recent years[2]. However, studies have shown that up to 40% of patients who achieve clinical and endoscopic remission still have persistent histological inflammation, which is associated with a higher risk of clinical recurrence of UC, receiving colectomy, and dysplasia[3].

Intestinal stem cells (ISCs) are important adult stem cells that drive the daily renewal of the intestinal epithelium through constant self-renewal, proliferation, and differentiation. ISCs are mainly located in intestinal recesses and play an important role in the repair of damaged intestinal mucosa[4]. In mammals, the gut consists of small villi that extend into the gut cavity and small intestine crypts that sink deep into the lining of the intestine. Leucine-rich repeat-containing G protein coupled receptor 5 (LGR5) is an important marker of ISCs[5]. Under the action of multiple signaling pathways in the body, LGR5-positive ISCs repair damaged intestinal mucosa and maintain intestinal homeostasis through self-renewal and differentiation potential. However, the internal mechanism of how multiple different signaling pathways interact with each other to synergistically regulate LGR5 cells with differentiation potential in UC remains unclear[6]. In this paper, the concept, location, quantity and cycle of ISCs, the repair mechanism of intestinal mucosa by ISCs, the renewal of colon epithelial cells and the regulation of nutritional molecules in damage repair were reviewed to further provide evidence-based medical evidence for the treatment objectives of UC.

CONCEPT, LOCATION, NUMBER AND CYCLE OF ISCS

Stem cells have the capacity for lifelong self-renewal. They are cells that can produce a variety of highly differentiated progeny and can respond differently to changes in their internal environment[7]. Morphologically, the cells at the bottom were counted as "one" in the longitudinal section of the lacunae. The ISCs were approximately located at the fourth layer of cells but fluctuated between the second layer and the seventh layer[8]. Stem cells have three levels of structure, each with different properties and functions. Stem cells have a long cycle. In general, stem cells undergo asymmetric division, but during development or after injury, they undergo symmetrical division and divide into two progeny stem cells to increase the number of stem cells. Normally, the excess stem cells produced by symmetrical division are eliminated by apoptosis or rapid differentiation[9]. After some lacunae die after toxic injury, such as radiation or chemotherapy, the remaining potential stem cells begin to exercise their stem cell potential and undergo symmetrical division to regenerate lacunae[10]. The lacunae also divide to produce more lacunae until the intestinal mucosa returns to normal.

ROLE OF ISCS IN INTESTINAL MUCOSA

Intestinal epithelial tissue is one of the most active self-renewing tissues in adult mammals. Intestinal epithelial cells renew every 5 d, and this process mainly depends on the continuous division and replenishment of ISCs. ISCs are a type of adult stem cell that are mainly distributed in the recesses of the intestine in mammals[11]. ISCs have asymmetric division, self-renewal, and pluripotency; that is, they proliferate and differentiate into a variety of cell types, including absorbent cells, goblet cells, intestinal endocrine cells, and Pan's cells. Each crypt of the intestinal mucosa contains 4 to 6 independent ISCs [12]. Morphologically, the count begins with cells at the base of the crypt, and the ISCs are located in the fourth layer of the crypt, where the stem cells have a very active cell cycle. ISCs first differentiate into transient extender cells, which are daughter cells with limited ability to divide and circulate[13]. The transient expansion cells settled at the base of the crypt for approximately 48 to 72 h, then gradually migrated upward, underwent approximately 6 rounds of cell division, and finally differentiated into terminal cells[14]. Studies have shown that small intestine recess stem cells can rapidly differentiate and repair damage in a small intestine radiation injury model under the action of insulin-like growth factor and hepatocyte growth factor[15]. Some scholars studied Drosophila intestinal mucosal damage induced by sodium glucan sulfate and found that the damaged intestine could secrete signaling proteins

to accelerate the division of ISCs to promote mucosal repair[16].

REPAIR MECHANISM OF ISCS ON INTESTINAL MUCOSA

Markers of ISCs

Each gut stem cell is coated with special protein receptors that selectively bind to or adhere to other "signaling" molecules. These cell surface receptors are known as stem cell markers. Currently, Musasi-1, telomerase reverse transcriptase (TERT) and ID14 are the main markers found in ISCs[17]. Musas-1 is a neural RNA-binding protein but has been shown to be a selective marker of ISCs in addition to the nervous system[18]. Some studies found that Musashi-1-positive cells were found in the small intestine of mice, and Musashi-1 was significantly increased in intestinal specimens of mice after reflex injury [19]. TERT is a ribonuclear protease complex. Studies have shown that immunohistochemical TERT-positive cells are mainly distributed in the base of the small intestine crypt, 4-7 cells away from the bottom of the crypt, and some cells are distributed in the interstitium surrounding the crypt[20]. ID14, a new gene found in Xenopus laevis, encodes a protein containing 315 amino acids[21]. Adult ID14 is mostly found in the intestine but is only weakly expressed in the stomach, lung and testis. Its expression in the intestine does not begin until the metamorphosis stage, which is closely related to the differentiation of adult intestinal epithelial cells[22].

Asymmetric division of ISCs

ISCs continuously increase the number of stem cells through asymmetric division to promote the self-renewal and repair of damaged intestinal tissues to maintain the dynamic balance of the intestinal mucosa[23]. Stem cells divide asymmetrically to form a daughter cell identical to the mother cell and a daughter cell capable of differentiation[24]. During this process of division, the stem cell DNA double strand tends to enter daughter cells that are identical to the mother cell so that the daughter cells that maintain the characteristics of the stem cell retain the mother strand DNA, thus maintaining the stability of the gene[25].

Neuroregulation of ISCs

Intestinal activity is innervated by the sympathetic, parasympathetic, and enteric nervous systems. The sympathetic and parasympathetic plexuses can promote the proliferation and regeneration of intestinal mucosal epithelial cells and accelerate the division of crypt cells through growth factors and inflammatory mediators[26]. The enteric nervous system consists of the intermuscular plexus and submucosal plexus, and most of its neurons are located in the intestinal wall[27]. It has been observed that chemical resection of the intestinal intermuscular nerve plexus can accelerate the proliferation of ISCs, indicating that the intermuscular nerve plexus has an inhibitory effect on intestinal mucosal cell renewal[28].

SELF-RENEWAL OF SMALL ISCS AND SMALL INTESTINAL EPITHELIUM

The intestinal epithelium is a single layer of cell epithelium covering the intestinal lining. As an important organ in mammals, the intestinal epithelium is responsible for digestion, absorption and resistance to intestinal pathogenic microorganisms[29]. Structurally, the epithelium of the small intestine is composed of a large number of repeating units called crypt villi[30]. The intestinal villi are composed of multiple differentiated cells that penetrate into the intestinal cavity to perform digestive and absorption functions, and the base of each villus encloses multiple intestinal recesses, each containing proliferative ISCs[31]. To avoid cytopathies caused by constant contact with external stimuli in the intestinal cavity, the small intestine epithelium is constantly renewing itself, and most cells renew themselves every 4-5 d on average. In line with this physiological function, small ISCs located in crypts have the ability of lifelong self-renewal, making the small intestinal epithelium an important model for adult stem cell research[32].

In the small intestine recess, small ISCs divide every 24 h on average, generating transient amplifying cells (TA cells) while renewing themselves[33]. Fast proliferating cells have a cell division cycle of approximately 12 h, migrating up the recess while performing several fast divisions[34]. In the process of upward migration, the descendant cells gradually differentiated into two types of cells, namely, the secretory lineage and the absorptive lineage. Secretory cells mainly include Paneth cells, goblet cells, and enteroendocrine cells, while absorptive cells mainly refer to intestinal epithelial cells. In contrast to the goblets, intestinal secretory cells and intestinal epithelial cells, which continue to migrate upward into the villi to perform their functions and reach the apex of the villi and undergo apoptosis within 3 to 5 d, Paneth cells migrate downward to the base of the crypt and survive for 3 to 6 wk[35].

Small ISCs and intestinal epithelial lesions

LGR5-labeled small ISCs not only mediate the normal self-renewal of the small intestine epithelium but

also act as the initiation cells of inflammatory cells in the case of mutation, seriously affecting life and health[36]. Since the self-renewal and repair rate of the small intestine epithelium is very fast, the imbalance of its renewal regulation easily leads to epithelial damage. LGR5-labeled small ISCs mediate the daily renewal of the small intestine epithelium, so the relationship between LGR5+ small ISCs and inflammation has received extensive attention[37].

Studies have shown that overactivation of the Wnt signaling pathway induces the release of inflammatory cytokines. Consistent with this, the vast majority of patients with UC carry the inactivated adenomatosis polyposis coli (*APC*) gene mutation. Using a mouse model, specific knockout of the APC gene in LGR5-labeled small ISCs resulted in a massive release of inflammatory cytokines in the short term[38]. Further studies showed that LGR5-positive cells in UC patients consistently produced all other cell types throughout the tumor tissue while self-renewing, demonstrating the tumor stem cell properties of LGR5-labeled cells[39].

Trophic molecular regulation of colon epithelial cell renewal and damage repair

The microenvironment refers to the surrounding environment where stem cells are located under physiological conditions and is usually composed of stem cells themselves, surrounding cells and the extracellular matrix[40]. Cell-to-cell contact in the microenvironment and the existence of various growth factors in the microenvironment coregulate the self-renewal and differentiation of stem cells [41]. LGR5+ small ISCs live in a specific environment, namely, at the bottom of the small intestine recess, mosaic among Pan's cells[42]. Paneth cells, TA cells, and peripheral mesenchymal cells together constitute a unique microenvironment for small ISCs, in which a variety of cell pathways, including the Wnt, Notch, epidermal growth factor (EGF), and bone morphogenetic protein (BMP) signaling pathways, cooperate to regulate the proliferation and differentiation of intestinal epithelial cells and repair after injury[43].

Wnt signaling pathway

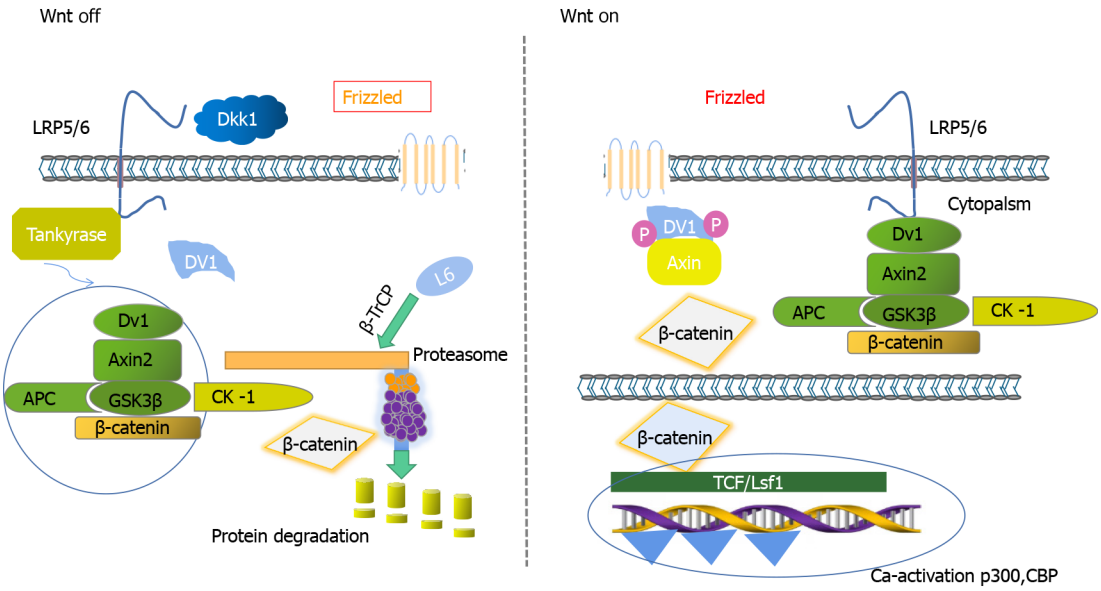
The Wnt signaling pathway is a highly conserved signaling pathway that regulates cell proliferation, cell fate determination and cell differentiation and plays a crucial role in embryonic development and adult stem cell maintenance[44]. Mutations in the Wnt signaling pathway are closely related to the occurrence of many diseases, especially colorectal cancer.

The Wnt signaling pathway plays a key role in the dry maintenance of small ISCs[45]. The first event that prompted the link of Wnt signaling to small ISCs was the discovery of a large number of *APC* gene mutations in colorectal cancer. As an important inhibitory factor of Wnt signaling, *APC* plays an important role in regulating Wnt signal strength. Mutation of the *APC* gene leads to overactivation of Wnt signaling[46]. Therefore, the overactivation of Wnt signaling may be closely related to the occurrence of colorectal cancer. In mouse models, *APC* gene mutation or deletion leads to the development of colorectal cancer. Both T cell factor 4 (*TCF4*) gene knockout and beta-catenin gene knockout will result in rapid loss of proliferative stem cell regions in the crypt[47]. All of this evidence suggests that activation of Wnt signaling promotes the dryness of small ISCs[48]. In line with this, Wnt signaling activity in the small intestinal epithelium decreased in a gradient along the crypt-villus axis, with the highest Wnt signaling activity at the base of the crypt[49]. The Wnt ligand is mainly secreted by Panzzled cells and peripheral mesenchymal cells at the base of the crypt. LGR5-labeled small ISCs actively express Frizzled receptors to transmit Wnt signals[50]. A series of target genes downstream of Wnt signaling mediate its physiological function. A large part of the abovementioned small ISC stem cells are direct target genes of Wnt signaling, including LGR5, achaete-scute family bHLH transcription factor 2, and Musashi-1. Other target genes of Wnt signaling, including Myc, play an important role in the occurrence of colorectal cancer[51] (Figure 1).

Notch signaling pathway

Notch signaling is a functionally conserved signaling family that exists widely in multicellular animals (metazoans). Notch signaling is mainly transmitted through cell-cell contact and plays an important role in physiological processes such as cell proliferation, stem cell maintenance, cell fate determination, differentiation and apoptosis[52].

Unlike most cellular pathway transduction processes, Notch signaling does not rely on a second messenger (secondary messengers). Posttranslational Notch protein is localized to the cell membrane as an active receptor after O-Fut-mediated glycosylation and PC5-mediated protease cleavage[53]. When ligands located near the cell membrane, such as Dll1, Dll4 and jagged1, bind to the Notch receptor, the Notch receptor is sequentially cleaved by ADAM and gamma-secretase-mediated protease[54]. The Notch receptor NICD (Noch intracellular domain) is released. Gamma-secretase-mediated protease cleavage may occur at the cell membrane or at the surface of endosome membranes containing NICDs, but NICDs produced by the latter usually enter the proteasome degradation pathway[55]. The released NICD is transferred into the nucleus, where it interacts with the DNA binding protein CSL (an acronym for C BF-1/RBPJ-κ in Homo sapiens / Mus musculus respectively, S uppressor of Hairless in Drosophila melanogaster, L ag-1 in Caenorhabditis elegans) and recruits a transcriptional coactivator to activate the expression of downstream target genes[56].



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Figure 1 Wnt signaling pathway. LRP5/6: LDL-receptor-related protein 5/6; Dvl: Dishevelled; APC: Adenomatosis polyposis coli; GSK-3β: Glycogen synthase kinase-3β; CK1: Casein kinase 1.

The Notch receptor is a single transmembrane protein that mainly includes Notch1, Notch2, Notch3 and Notch4 members in mammals. The Notch receptor extracellular end contains 29 to 36 EGF-like repeats, which may mediate Notch receptor and ligand interactions[57]. In mammals, Notch signaling ligands also contain multiple members, including Jagged1, Jagged2, Dll1, Dll3 and Dll4. The interaction of multiple ligands with multiple receptors increases the complexity of the Notch signaling pathway[58] (Figure 2).

BMP signaling pathway

BMP is a transforming growth factor (TGF-β). TGF-β is an important member of the TGF-β superfamily [59]. By regulating the activity of downstream genes, they play an important role in mesoderm formation, nervous system differentiation, bone development and cancer occurrence. BMP signal transmission occurs mainly through the specific binding of BMP protein to the BMP receptor (BMPR) on the cell membrane. Meanwhile, regulated Smads (R-SMAD) are regulated by activated type I receptors (BMPR1), which detach Smad molecules from cell membrane receptors[60]. After binding Smad4 [Common-mediator Smad (Co-SMAD)] in the cytoplasm, it enters the nucleus and coregulates the transcription of target genes with the participation of other DNA-binding proteins[61].

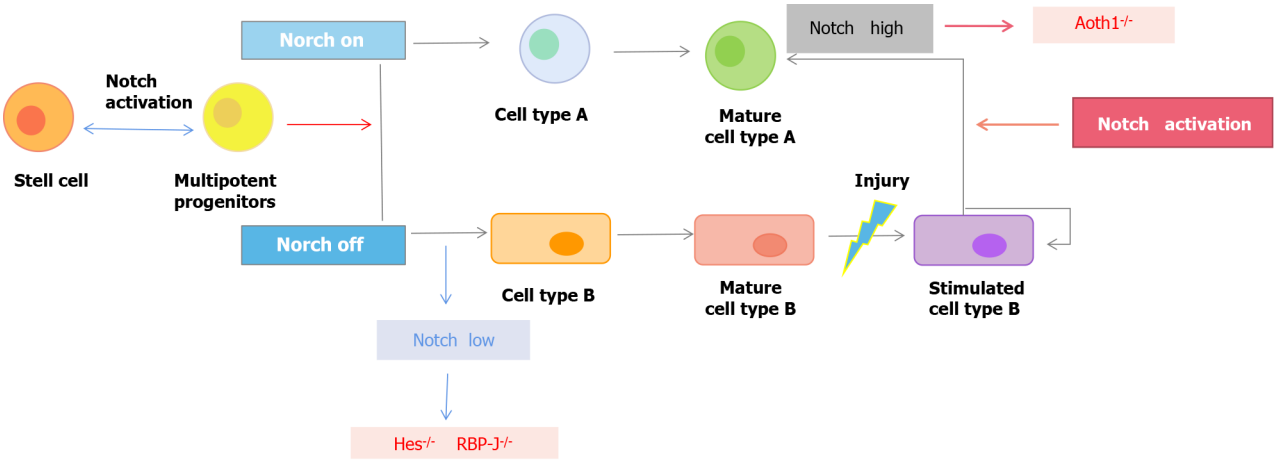
In contrast to Wnt signaling activity, BMP signaling activity increased gradually along the crypt-villus axis. In the small intestine, BMP ligands, including BMP2 and BMP4, are mainly secreted by mesenchymal cells around the crypt and inside the villi, while the BMP receptor Bmpr1a is expressed throughout the small intestine epithelium. Because peripheral mesenchymal cells also secrete BMP ligand inhibitors, including Noggin and Gremlin1, the BMP signal intensity in the crypt is low[62].

Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway is essential for embryonic development and cell growth and differentiation after embryogenesis[63]. Among mammals are three Hh family members: Sonic Hh, Indian Hh (Ihh), and Desert Hh. Ihh is the main Hh protein expressed in the intestine. It acts on mesenchymal cells through paracrine signaling by differentiated epithelial cells and negatively regulates the proliferation of crypt columnar cells by increasing BMP signals[64]. In addition, Ihh inhibits the lamina propria immune response. Without causing any damage to the upper cortex, Ihh knockout activates an immune response similar to the wage-healing response, epithelial remodeling, and recruitment of fibroblasts and macrophages[65]. Therefore, the decreased expression of Ihh caused by the injury or dysfunction of the upper cortex, thus triggering the damage repair of the interstitial cells, may be one of the main mechanisms of the wound healing response[66].

Hippo-YAP signaling pathway

The Hippo signaling pathway is a newly discovered cell signal transduction pathway whose main part is a kinase chain. Among them, kinase MST1/2 (mammalian Sterile 20-like kinases 1/2) can phosphorylate and activate LATS1/2 (large tumor suppressor 1/2)[67]. LATS1/2 phosphorylates and inhibits the key kinase Yes-associated protein (YAP)/Tafazzin (TAZ). YAP/TAZ are two homologous



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Figure 2 Notch signaling pathway.

transcription cofactors that mediate most of the physiological and pathological functions of Hippo signaling pathways[68]. YAP is expressed in both the small intestine and large intestine, with low expression in the small intestine but high expression in the colon, especially in the terminal colon. At the cellular level, YAP was localized in the cytoplasm in intestinal villi and upper crypt cells and in the nucleus in LGR5+ ISCs at the bottom of crypts and was expressed at low levels in Pan's cells, indicating that YAP activity was negatively correlated with the degree of differentiation of intestinal epithelial cells [69].

The Hippo signaling pathway plays an important role in regulating the differentiation of ISCs. The proliferative ability of mouse intestinal epithelial-specific YAP transgenic stem cells increased, while the differentiation ability decreased[70]. Consistently, MST1/2 knockout in the mouse gut promoted stem cell proliferation, accompanied by abnormal crypt cell differentiation and reduced goblet cells[71]. Some studies have found that LATS1/2 double knockout in the intestine promotes the proliferation of crypt cells, an increase in ISCs, and the differentiation of goblet cells[72]. Further study showed that YAP/TAZ could cooperate with Klf4 to promote the differentiation of crypt cells into goblet cells. Regarding the effect of Hippo signaling pathway inhibition on goblet cell differentiation, the results of the above two experiments were different. Some scholars believe that this is because the intestine-specific gene transfer method they used can mildly express YAP exogenically or inhibit MST1/2 and LATS1/2, so that YAP can not only promote ISC proliferation but also cause differences in the activity of goblet cell differentiation. These results indicate that the regulatory effect of YAP on ISCs is closely related to its activity level[73] (Figure 3).

UC INTESTINAL INFLAMMATORY RESPONSE PROCESS

Intestinal mucosal mucus secretion decreased

The intestinal mucosa is covered with a thick layer of mucous substances containing a variety of antimicrobial molecules, which can play a protective role in the intestinal mucosa. It can lubricate the intestine and resist the invasion of microorganisms, pathogens and other harmful substances, acting as a chemical barrier and mechanical barrier protection[74]. One possible reason for the aggravation of UC is that the number of goblet cells in the intestinal mucosa is reduced, and the function of intestinal mucus secretion is impaired. Colon mucosal epithelial cells are mainly composed of goblet cells (GCs), secretory cells that secrete a large number of mucoproteins (MUCs) and intestinal trefoil factors (ITFs) and human intestinal resistin-like molecule β (resistin-like molecule β, RELM-β)[75].

The main component of the mucin layer is mucin, which is a high molecular weight glycoylated protein secreted by GCs. It is an important bioactive peptide that can coat bacteria and prevent direct contact between bacteria and epithelial cells[76]. Therefore, mice with insufficient mucus secretion easily develop UC, and studies have shown that MUC2-deficient mice or MUC2 gene mistranslation mice can spontaneously develop UC[77].

Studies have shown that the synthesis of MUC2 in the colon during MUC activity is 40% less than that in the normal colon, indicating that the decrease in mucin in the colon mucosa is one of the reasons for the weakening of intestinal mucosal barrier function and the pathogenesis of UC[78]. The Notch signaling pathway is one of the important ways to maintain the proliferation and differentiation of colon epithelial cells. Overactivation of Notch leads to increased expression of the transcription factor HE-1 in human colon cell lines, inhibits Hath-1 expression, and subsequently inhibits the differentiation

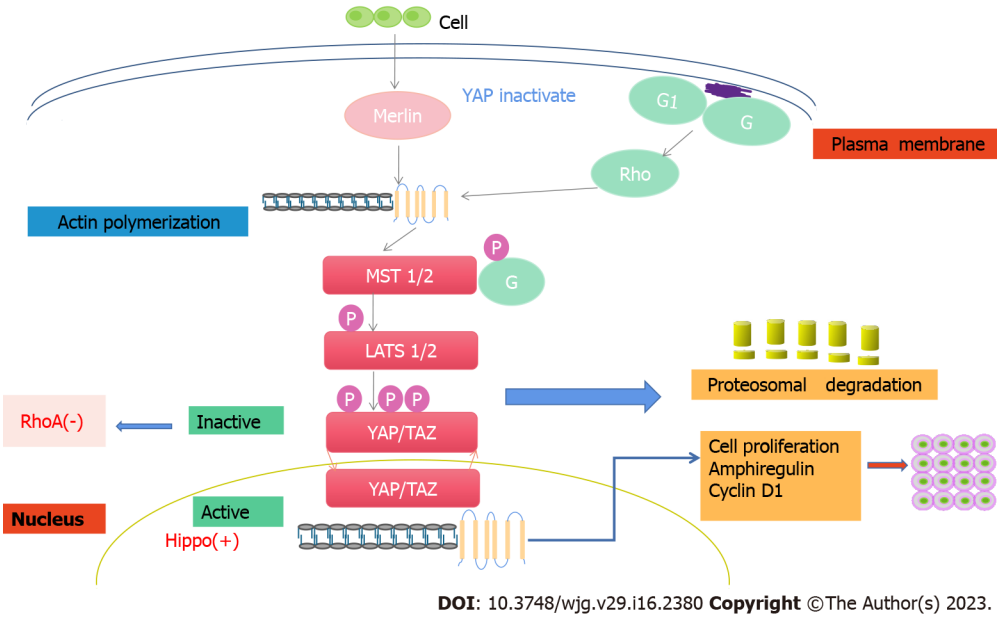


Figure 3 Hippo signaling pathway. MST: Mammalian sterile; LATS: Large tumor suppressor; YAP: Yes-associated protein; TAZ: Tafazzin.

of intestinal epithelial cells into goathous cells, resulting in a decrease in secretory cells and formation of the intestinal mucosal layer[79].

Intestinal mucosal oxidative stress response

Chronic intestinal inflammation can cause a large number of white blood cells to infiltrate the intestinal mucosa, including neutrophils and macrophages, which can produce inflammatory factors and excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing an intestinal mucosal oxidative stress response and intestinal mucosal damage together with an inflammatory response[80]. ROS content was positively correlated with the occurrence and development of UC. ROS consist of a variety of components, including peroxide, hydroxyl and a large amount of hydrogen peroxide. RNS include nitric oxide, nitrogen dioxide and peroxynitrite[81].

When UC occurs, colon mucosa produces a large number of inflammatory molecules and activates a large number of inflammatory response pathways, which jointly promote the production of a large number of peroxides and accumulate in the intestine, self-sustaining and amplifying intestinal oxidative stress, forming a vicious cycle[82]. A large number of ROS can destroy the structure of intestinal endothelial cytoskeleton proteins and cause intestinal mucosal barrier dysfunction. Finally, the structure and function of the intestinal mucosal barrier are damaged, which affects the protective effect of the intestinal tract[83]. ROS can also increase the permeability of the cell membrane. On the one hand, ROS can cause the inflow of extracellular Ca^{2+} into the cell to promote the apoptosis of intestinal cells; on the other hand, ROS can cause a peroxidation reaction with the cell membrane to damage the normal structure and function of intestinal mucosal cells and further lead to the impairment of intestinal mucosal function[84].

Intestinal mucosal barrier damage

The intestinal mucosal barrier has selective permeability. When the intestinal mucosal barrier is destroyed, mucosal inflammation can cause necrosis and shedding of epithelial cells, which increases intestinal mucosal permeability[85]. Structural damage to epithelial cells leads to changes in the tight connective structure and loss of protective effects so that various pathogenic substances in the intestinal cavity are absorbed into the body[86]. The intestinal immune system is repeatedly stimulated and misidentified with these harmful substances, which continuously activates intestinal macrophages and tissue lymphocytes and further stimulates or aggravates the release of inflammatory factors in the intestine, thus continuously initiating an excessive intestinal immune inflammatory response and ultimately damaging the intestinal mucosal barrier, resulting in the loss of protective function of the intestinal mucosal barrier and damage to intestinal tissues[87]. A large number of studies have shown that a large number of epithelial cells in the inflammatory site of the intestinal mucosa in UC patients suffer from apoptosis, and the resulting tight connection injury is considered to be an important cause of UC[88].

It was found that the goblet cells and mucus secreted by intestinal mucosa in patients with UC were reduced. Tight junctions are occlusive links formed by the binding of the outer layer of the adjacent intestinal epithelial cell membrane by specific transmembrane proteins[89]. Tight junctions are mainly

composed of tight junction proteins, including Occludin, the claudin family, Zonula occludens (ZO), the ZO family and junctional adhesion molecule (JAM), which are important structures of epithelial barrier function and play a decisive role in intestinal mucosal permeability by JAM[90]. As a transmembrane tight junction protein, Occludin can form the paracellular tight junction structure and is an important structural and functional protein involved in signal regulation of tight junction formation. Studies have shown that the silencing of occludin genes can increase the cell bypass permeability of intestinal epithelial cells, resulting in an increase in macromolecules and harmful substances in the intestine[91]. Several studies have shown that occludin gene expression in the colon mucosa of UC patients decreases, resulting in a decrease in occludin protein synthesis[92]. Claudins are one of the transmembrane proteins of intestinal epithelial cells. The extracellular part of Claudins acts as a ligand and interacts with transmembrane lectin receptors of adjacent epithelial cells to bind, thus filling the cellular gap and maintaining the tight connection function of the intestinal mucosa[93]. ZOs act as an "assembly platform" for tight junctions that link transmembrane proteins and the cytoskeleton to recognize and transmit signals[94]. The decreased expression of ZOs in intestinal mucosal epithelial cells indicated increased intestinal permeability and damage to the intestinal mucosal barrier[95]. Tight junctions are regulated mainly by protein phosphorylation. When the intestinal mucosa is stimulated by inflammation or oxidative stress, Occludin and ZO-1 phosphorylation are deactivated, and reallocation of the Occludin-Zo-1 complex affects the normal structure of tight junctions of intestinal epithelial cells, resulting in increased intestinal permeability and damage[96].

LGR5-labeled small ISCs

LGR5-labeled small ISCs are the most recognized small ISCs. The LGR5-labeled cells are located at the bottom of the crypt base columnar cell (CBC), which is also called the crypt base CBC because of its small size and elongated shape[97]. As early as 1974, the CBC stem cell model was proposed. According to the theory, CBC cells are small ISCs that live in a microenvironment formed by Paneth cells[98]. Once their offspring leave this microenvironment, they begin to differentiate into a variety of differentiated cells[99]. It was not until 2007, when the CBC cell-specific marker LGR5 was identified, that the theory was experimentally confirmed[100]. In the LGR5-enhanced green fluorescent protein (EGFP)-IRES-Cre ERT2 gene knockout mouse model, CBC cells were labeled with EGFP fluorescent protein. Lineage tracing experiments subsequently demonstrated that the progeny of CBC cells could differentiate into any kind of cell in the small intestinal epithelium, and such lineage markers could persist in the small intestinal epithelium, demonstrating the small ISCs property of CBC cells. EGFP fluorescent protein-labeled CBC cells were isolated using flow cytometry and were encapsulated in Matrigel for *in vitro* stem cell culture in the presence of three growth factors (EGF, Noggin, and R-spondin). Individual LGR5+ cells can grow into organoids, which closely resemble the structure and cellular composition of the intestinal epithelium in vivo. LGR5+ small ISCs can both self-renew and generate all progeny differentiated cells. This evidence suggests that LGR5-labeled cells represent small ISCs[101].

LGR5-labeled small ISCs are actively dividing stem cells that divide every 24 h on average. LGR5+ cells produce transient multiplication cells while generating new small ISCs[102]. TA cells migrate upward and differentiate gradually during subsequent rapid division. The present study suggests that self-renewal of LGR5+ small ISCs follows a "neutral competition" model. LGR5+ small ISCs can maintain their dry properties only when they are located in a microenvironment composed of Pan cells [103]. Because LGR5+ small ISCs divide symmetrically, the progeny cells forced out of the microenvironment due to space crowding will differentiate into TA cells, while the progeny cells left in the microenvironment will retain their stem cell properties[104].

The "+ 4 stem cell" model is another theory about the localization of small ISCs. The + 4 cells refer to the cells placed fourth from Paneth cells at the bottom of the crypt and are considered small ISCs because of their label retention ability[105]. Marker retention means that after marking the DNA of +4 cells, these markers remain in + 4 cells for a long time afterward and do not disappear with cell division [106]. This marker retention property is often thought to be unique to stem cells. At present, markers of + 4 stem cells have been identified, including Bmi and Lrig1, Hopx and Tert, *etc*. However, the specificity of these markers has been under great controversy. Studies have shown that cells at the bottom of the small intestine recess all express these genes; that is, the expression of these genes is not substantively specific[107].

The role of LGR5 and BMP pathways in UC mucosal injury

Significant expansion of LGR5+ small ISCs was detected under normal physiological conditions after BMP signaling was blocked by directly inducing the small intestinal epithelial receptor Bmpr1a, which specifically knocked out BMP signaling[108]. Specific knockout of the Bmpr1a receptor in LGR5+ small ISCs also led to rapid expansion of stem cell groups. In vitro culture and *in vivo* lineage tracing experiments showed that the self-renewal and proliferation abilities of LGR5+ small ISCs were significantly enhanced after BMP signaling inactivation[109]. In the case of long-term BMP signal inactivation, continuous and unrestricted expansion of LGR5+ small ISCs will lead to malignant proliferation of the small intestinal epithelium and the appearance of small intestinal polyps[110]. These phenotypes are very similar to the symptoms of human juvenile intestinal polyps. Finally, the radiation damage model also verified the upregulation of stem cell function after BMP signal inactivation[111].

That is, after BMP signal inactivation, a certain dose of radiation damage can only lead to the apoptosis of some LGR5+ small ISCs, while the remaining LGR5+ small ISCs actively participate in the process of damage repair, thus greatly accelerating the process of radiation damage repair[112].

The relationship between BMP signaling inactivation and self-renewal disturbances in the small intestine epithelium has long been noted. This is because inactivated mutations in the BMP signaling pathway, including the BMPR1A and SMAD4 genes, are found in most human genetic juvenile polyps [113]. In the small intestine, the BMP ligands BMP2 and BMP4 are mainly secreted by mesenchymal cells in the intestinal villi and mesenchymal cells around the small intestine recess. The BMP inhibitors Noggin and Gremlin1 are mainly secreted by mesenchymal cells around the crypts of the small intestine [114]. This secretion pattern results in higher BMP signaling activity in the villi and lower BMP signaling in the crypts of the small intestine. Similarly, however, cells in the mesenchyme responding to BMP stimulation should also be in a state of BMP signaling activation due to the abundance of BMP ligands in the mesenchyme[115]. In juvenile intestinal polyps, BMP signaling inactivation means that all cells no longer respond to BMP signaling, and malignant proliferative intestinal polyps appear[116]. Therefore, it is not clear whether BMP signaling in epithelial cells or in mesenchymal cells plays an important role in inhibiting the appearance and growth of small intestinal polyps. Earlier studies using a Noggin transgenic overexpression mouse model and a systemic Bmpr1a knockout mouse model failed to solve this problem[117].

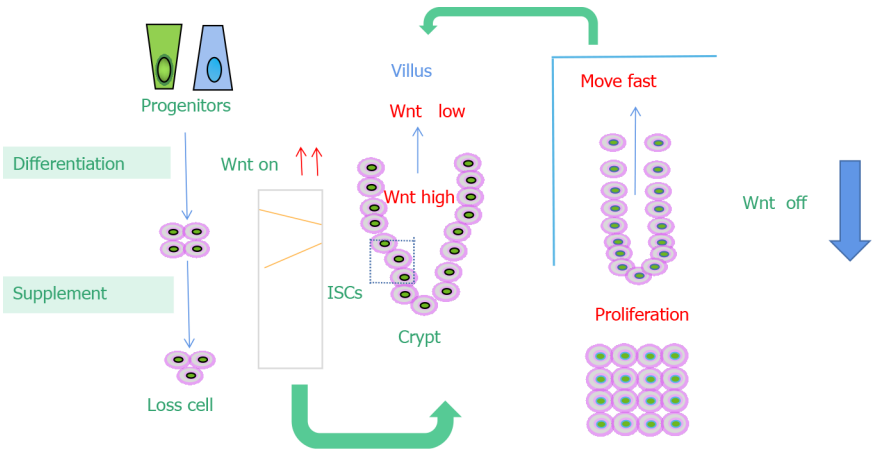
The Wnt/β-catenin signaling pathway promotes ISC proliferation and maintains intestinal epithelial homeostasis

In the small intestine, the Wnt/β-catenin signaling pathway is thought to be critical for maintaining ISCs self-renewal and proliferation. Wnt was highly expressed in the stem cell area and around proliferating cells in the small intestine, and it decreased gradientally upward with the intensity of differentiation. Genes expressed in intestinal epithelial stem/progenitor cells, such as those that label ISCs LGR5 and Olfm4, are regulated by Wnt signals[118]. There are 19 different Wnt genes expressed in the small intestine. The main cell source of classical Wnt, such as Wnt3, Wnt6 and Wnt9b, is epithelial cells, not classical Wnt[119]. For example, Wnts2b, Wnt4, Wnt5a, Wnt5b, and Wnt antagonists [secreted frizzled related protein (SFRP)-1, SFRP-2, Dickkopf (DKK)2, and DKK-3] are derived from mesenchymal cells. Wnt secreted by epithelial or stromal cells first binds to the coreceptors LRP5/6 and Frizzled on the cell membrane in crypt cells, causing increased expression of β-catenin. Activated beta-catenin further binds to the nuclear transcription factor TCF4 to drive gene expression that supports stem cell maintenance, proliferation, and differentiation[120]. Blocking the Wnt/β-catenin signaling pathway leads to the stagnation of intestinal epithelial cell proliferation. Previous studies have demonstrated that knockout of TCF, DKK1 (Wnt antagonist), Ctnnb1 (β-catenin gene), or c-Myc (Wnt target gene) can significantly affect the proliferation of intestinal epithelial cells in mice. TCF4 knockout in embryonic intestinal epithelial cells resulted in no proliferation in the intervillus region of the small intestine in neonatal mice, while induced knockout of TCF4 and Ctnnb1 blocked crypt proliferation in adult mice[121]. In contrast, the addition of the Wnt agonist R-spondin (roof plate-specific spondin) or the elimination of APC resulted in small intestine or colorectal hyperplasia. Meanwhile, the deletion of Wnt key mediators ring finger protein 43 and zinc and ring finger 3 will also cause intestinal proliferation. Therefore, Wnt signaling plays an important role in the dry maintenance, proliferation and differentiation of small ISCs [122].

Although a large number of studies have confirmed that Wnt secreted by interstitial cells is essential in small intestine development, formation, and damage repair, the evidence that secreted Wnt regulates small intestine homeostasis in mice remains unclear[123]. Wnt3 derived from Pan's cells is necessary for the *in vitro* culture of LGR5-labeled ISCs organoids. Other studies have shown that Wnt generated from epithelial or mesenchymal cells supports intestinal epithelial growth in organoids in vitro[124]. Some scholars have demonstrated that purified stromal cells can support the formation of epithelial organoids that knock out Wnt3. However, mouse models with Paneth cells removed or Wnt3 knocked out in intestinal epithelial cells showed no obvious phenotype, and the types of interstitial cells secreting Wnt in the *in vivo* small ISCs microenvironment, as well as the mechanism of inducing secretion, remain unclear, so more *in vivo* evidence is needed to provide support[125] (Figure 4).

The Notch signaling pathway guides the differentiation of ISCs

Notch receptors and ligands are expressed only at the crypt site, and their signaling activity plays an important role in the self-renewal and differentiation of the small intestine epithelium[126]. First, Notch signaling regulates the differentiation process of small ISCs. In the process of upward migration, TA cells gradually differentiate into two types of cells, namely, secretory cells and absorptive cells. This differentiation process is mainly regulated by the Notch signaling pathway. Notch activation inhibited cell differentiation toward the secretory type and promoted cell differentiation toward the attractor type. Specific inhibition of Notch signaling in the small intestinal epithelium can rapidly transform all small intestinal crypt cells into secretase cell types by Notch receptor, ligand knockout, or gamma-secretase inhibitor treatment[127]. Conversely, activation of Notch signaling in the small intestinal epithelium significantly inhibited secretory cell differentiation. Second, Notch signaling regulates the



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Figure 4 The Wnt/β-catenin signaling pathway promotes intestinal stem cells proliferation and maintains intestinal epithelial homeostasis. ISCs: Intestinal stem cells.

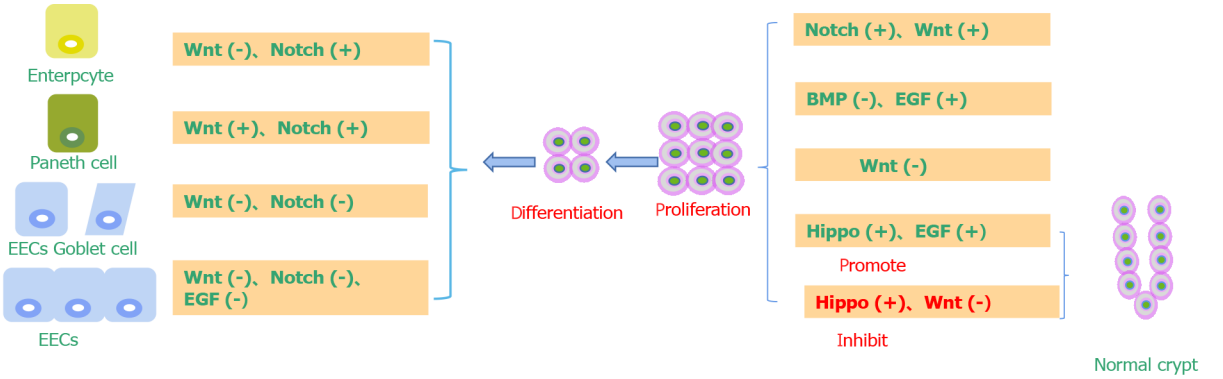
self-renewal of small ISCs. In the small ISCs microenvironment, the ligands for Notch signaling are mainly provided by Paneth cells, and Notch receptors are actively expressed in small ISCs[128]. Using the mouse model, cells with high Notch signaling activity were specifically labeled. Using lineage tracing experiments, it was found that small ISCs belong to a type of cell with high Notch signaling, and these cells can form all cell types in the small intestine epithelium[129].

Interaction between the Wnt pathway and Notch pathway

The Wnt and Notch signaling pathways are two highly conserved signal transduction pathways that exist widely in multicellular animals. They regulate many life processes through different mechanisms and play an important role in cell proliferation, differentiation, and intestinal homeostasis[130]. However, the specific mechanism by which these two signaling pathways interact to regulate ISC activity and differentiation direction remains unclear. A large number of studies have reported crosstalk between Wnt and Notch signaling pathways[131]. The mechanisms are discussed as follows: (1) Wnt protein regulates downstream through binding to some Notch receptors, including Dfrizzled2, patched, shaggy, *etc.*, hairy and patched genes are expressed, and Dfrizzled2 and patched genes can mediate the Wnt pathway itself[132]; (2) Dvl can antagonize the Notch pathway through its direct interaction with Notch intracellular domain (NIC)[133]; (3) NIC can increase the activation potential of lymphoid enhancer factor under the action of some promoters[134]; (4) GSK-3β phosphorylates NIC, prevents its degradation by the proteasome, and prolongs its half-life[135]; and (5) C promoter binding factor-1 can promote the expression of some genes encoding Fz[136]. In addition to the direct crossover between pathways, there are also many indirect (for example, some pathways in both pathways are involved in the regulation of cyclin D1 and p21 expression) and mechanistic associations[137] (Figure 5).

CONCLUSION

In conclusion, intestinal mucosal injury is an important pathological change in UC. ISCs proliferation and differentiation are the main cytological basis for intestinal mucosal renewal. ISCs participate in normal physiological processes and some pathological processes of the intestine. They are located at the base of the crypts of the intestinal mucosa, which is the cell bank of ISCs. All cells of the intestinal epithelium were derived from crypt stem cells. Meanwhile, LGR5-positive ISCs are significantly regulated by the Notch signaling pathway and Wnt/β-catenin signaling pathway, which jointly maintain the function of LGR5-positive ISCs. More importantly, the surviving stem cells after intestinal mucosal injury accelerate division, restore the number of stem cells, multiply and differentiate into mature intestinal epithelial cells, and repair the damaged intestinal mucosa. Therefore, in-depth study of multiple pathways and transplantation of LGR5-positive ISCs may become a new target for the treatment of UC.



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Figure 5 Interaction between the Wnt pathway and Notch pathway. EGF: Epidermal growth factor; BMP: Bone morphogenetic protein.

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FOOTNOTES

Author contributions: Zheng L contributed to the conception and design of the study and performed to the writing of the manuscript; Zheng L and Duan SL contributed to literature search, drafting of the manuscript, and providing approval of the final version to be published.

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REVIEW

SARS-CoV-2 induced liver injury: Incidence, risk factors, impact on COVID-19 severity and prognosis in different population groups

George D Liatsos

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Abstract

Liver is unlikely the key organ driving mortality in coronavirus disease 2019 (COVID-19) however, liver function tests (LFTs) abnormalities are widely observed mostly in moderate and severe cases. According to this review, the overall prevalence of abnormal LFTs in COVID-19 patients ranges from 2.5% to 96.8% worldwide. The geographical variability in the prevalence of underlying diseases is the determinant for the observed discrepancies between East and West. Multifactorial mechanisms are implicated in COVID-19-induced liver injury. Among them, hypercytokinemia with "bystander hepatitis", cytokine storm syndrome with subsequent oxidative stress and endotheliopathy, hypercoagulable state and immuno-thromboinflammation are the most determinant mechanisms leading to tissue injury. Liver hypoxia may also contribute under specific conditions, while direct hepatocyte injury is an emerging mechanism. Except for initially observed severe acute respiratory distress syndrome corona virus-2 (SARS-CoV-2) tropism for cholangiocytes, more recent cumulative data show SARS-CoV-2 virions within hepatocytes and sinusoidal endothelial cells using electron microscopy (EM). The best evidence for hepatocellular invasion by the virus is the identification of replicating SARS-CoV-2 RNA, S protein RNA and viral nucleocapsid protein within hepatocytes using in-situ hybridization and immunostaining with observed intrahepatic presence of SARS-CoV-2 by EM and by in-situ hybridization. New data mostly derived from imaging findings indicate possible long-term sequelae for the liver months after recovery, suggesting a post-COVID-19 persistent live injury.

Key Words: COVID-19; SARS-CoV-2; Liver injury; Cytokine storm; Endotheliopathy; Immuno-thromboinflammation; Direct hepatocyte injury

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Core Tip: Following respiratory system, liver is the second most involved organ in coronavirus disease 2019 (COVID-19). Besides the well-observed cholangiocyte tropism, typical severe acute respiratory distress syndrome corona virus-2 (SARS-CoV-2) Lesions indicated by ultrastructural and histological evidence, identification of replicating SARS-CoV-2, S and nucleocapsid proteins RNAs within hepatocytes, as well as intrahepatic virus observation by electron microscopy and in-situ hybridization, converge to the conclusion that SARS-CoV-2 may also be hepatotropic. Most prevalent mechanisms of COVID-19-related liver injury are hypercytokinemia with "bystander hepatitis", cytokine storm syndrome with subsequent oxidative stress, endotheliopathy and immuno-thromboinflammation. Depending on the grade of their abnormalities, increased serum aspartate aminotransferase, (mostly peak) alanine aminotransferase, alkaline phosphatase, total bilirubin, inflammatory markers (C-reactive protein, ferritin, interleukin-6, -10) and decreased albumin levels are independent discriminators of COVID-19 severity and mortality. Age, male gender, chronic liver disease, liver cirrhosis, obesity, diabetes, and non-alcoholic fatty liver disease are independent prognostic factors of unfavorable COVID-19 outcomes.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA determined by quantitative rt-polymerase chain reaction is widely spread outside the respiratory tract, including the liver[1]. Regardless of pre-existing chronic liver disease (CLD), coronavirus disease 2019 (COVID-19)-induced liver injury (LI) is mainly reflected by hypertransaminasemia, elevations of gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) (less frequently), and hypoalbuminemia[2-8], with the later being a negative acute phase reactant rather than manifestation of liver failure and is one of the most prevalent abnormalities. COVID-19-induced LI is secondary than primary[9,10], mostly mild, transitory and self-limiting[11], it does not impact the majority of patients[12], and is common in absence of CLD[13]. In asymptomatic/subclinical cases randomly diagnosed by computed tomography (CT) scans a mild increase in transaminases (8.8%) is observed[14]. LI definition varies among values just above the upper limit of normal (ULN)[15,16] up to 2–5 × ULN[17,18]. Substantial transaminases increases are linked to unfavorable outcomes, such as death, invasive mechanical ventilation (IMV), and intensive care unit (ICU) admission[7,18-22]. The prognostic relevance of higher liver function tests (LFTs) may result from a more vigorous host immunological and inflammatory response to infection[12], particularly in younger individuals[12,23]. The pattern of LI is typically hepatocellular rather than cholestatic[24]. Severe LI (SLI) defined as alanine aminotransferase (ALT) elevations > 10–15 × ULN with or without jaundice, occurs in 2% of COVID-19[25], while acute liver failure (ALF) without underlying CLD is extremely rare and is typically associated with severe pneumonia and multiple organ dysfunction syndrome (MODS)[26]. SARS-CoV-2-induced ALF has been described in case reports[21].

PATHOPHYSIOLOGY OF SARS-COV-2 INFECTION

The SARS-CoV-2 spike (S) protein is recognized by the angiotensin converting enzyme-2 (ACE2), whilst the androgen-induced transmembrane serine protease-2 (TMPRSS2) and paired basic amino acid cleaving enzyme (FURIN) are necessary for cell tropism and entry[27,28]. ACE2 cleaves the vasoconstrictor peptide angiotensin II to vasodilator angiotensin I[29]. S protein interacts with ACE2[30] and with an identified co-receptor neuropilin-1[31]. FURIN evades immune surveillance thus promoting transmission[28]. SARS-CoV-2 binding to ACE2 causes inflammation, oxidative stress, and pro-apoptotic reactions, ultimately leading to LI[30]. According to single-cell RNA sequencing studies of healthy livers, cholangiocytes exhibit the highest expression of ACE-2, with modest expression found in hepatocytes, sinusoidal endothelial cells, and resident Kupffer cells[32]. Luminal immunohistochemical staining for ACE2 is observed in the bile ducts[33]. A few hepatocytes co-express both TMPRSS2 and FURIN[34,35]. Liver ductal organoids that express ACE2 and TMPRSS2 have been shown to recapitulate SARS-CoV-2 infection[36], whereas liver organoids generated from pluripotent stem cells also express ACE2 and allow SARS-CoV-2 pseudoparticle entry[36,37]. A small population of TROP2+ liver epithelial progenitors express both ACE2 and TMPRSS2. In healthy livers *vs* cirrhotics, 1.8/10000 cells *vs* 10.6/10000 expressed ACE2 and 97.2/10000 *vs* 216/10000 expressed TMPRSS2 representing a significant ($P < 0.001$) increase in the number of TMPRSS2+ cells in cirrhotics[38]. In untreated hepatitis B virus

(HBV) infected livers, only 1.4/10000 and 48.3/10000 cells expressed ACE2 and TMPRSS2 respectively, significantly fewer than both healthy and cirrhotics[38]. ACE2 expression is 30 times higher in hepatitis C virus (HCV)-related cirrhosis than in healthy liver[39]. As ACE2 has been identified as an interferon-inducible gene[40,41], LI and inflammation may therefore enhance SARS-CoV-2 hepatotropism by modifying viral receptor expression, which is consistent with the damage to the respiratory epithelia [41]. In non-infected individuals with obesity and Non-alcoholic Steatohepatitis (NASH), ACE2 and TMPRSS2 Liver mRNA co-expression is likewise upregulated[42]. Non-alcoholic fatty liver disease (NAFLD) and cirrhotic livers have much higher TMPRSS2+ progenitor cells indicating a susceptibility to SARS-CoV-2, findings consistent with the sc-RNA-seq results[38]. Given the recognized link between obesity and NAFLD[43], the finding of a larger abundance of TMPRSS2+ progenitor cells in NAFLD livers may offer a potential explanation for why obese people experience more severe COVID-19[38].

RATIONALE AND MECHANISMS OF LIVER INJURY

The variability in prevalence and severity of LI among COVID-19 patients suggests that the mechanisms of LI are multifactorial (Figure 1).

Direct liver injury

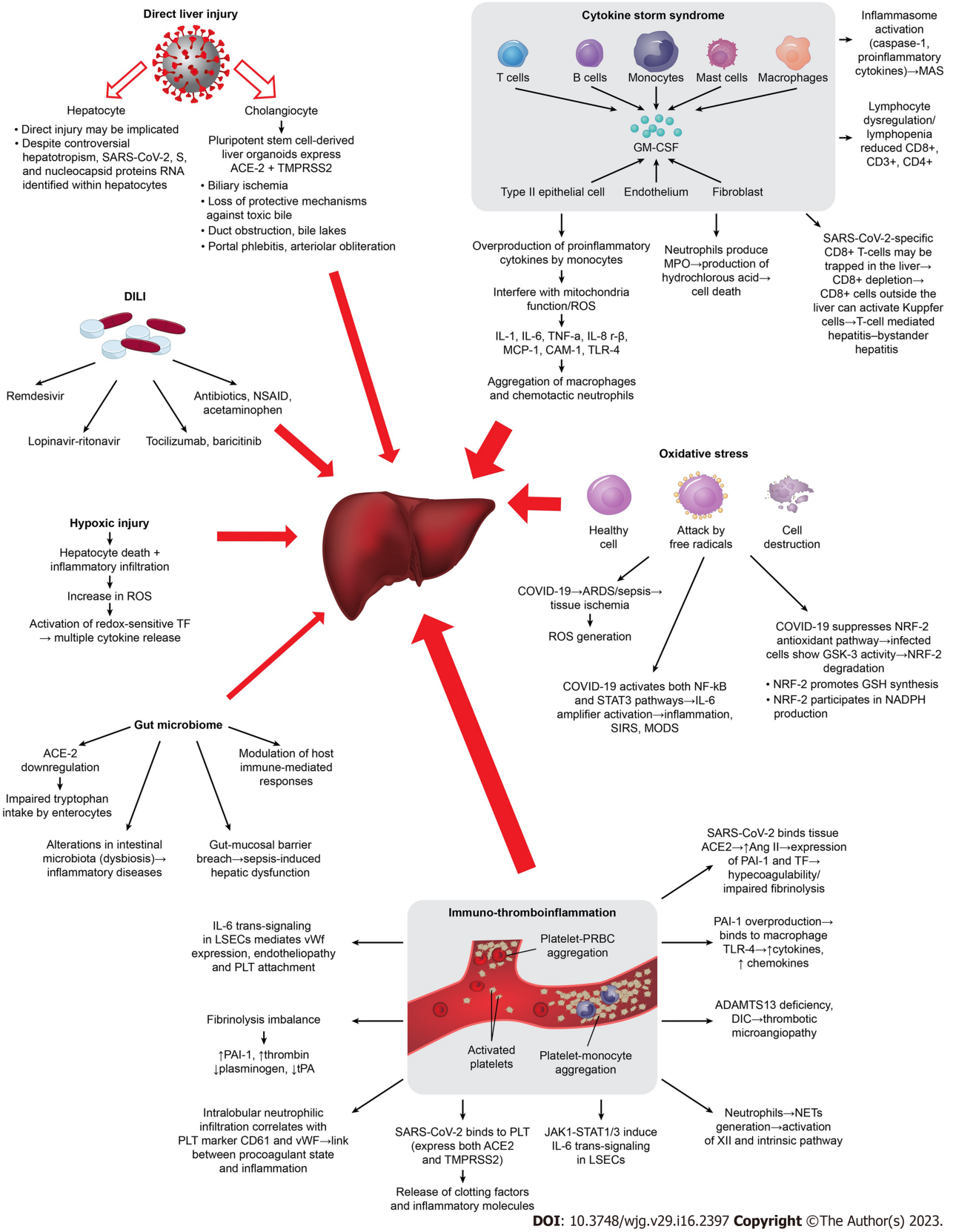
Many studies propose that SARS-CoV-2 hepatotropism and its direct liver function impairment is implicated in COVID-19-induced LI, while only a few speculate that definite evidence is lacking[44,45]. Liver progenitor cells, particularly those destined to become cholangiocytes, contain ACE2[46], in addition to virus isolation in bile[47] imply a direct invasion by SARS-CoV-2. Its infection triggers cell apoptosis factors resulting in cholangiocyte death[36] by lysis and/or by inducing necrosis and apoptosis[48-50]. SARS-CoV-2 virions have been seen within hepatocytes and sinusoidal endothelial cells using electron microscopy (EM)[51]. The best evidence is the identification of replicating SARS-CoV-2 RNA, S, and nucleocapsid proteins RNA within hepatocytes using in-situ hybridization and immunostaining[51,52]. In hepatocytes and sinusoidal endothelial cells, SARS-CoV-2 virions have been seen using EM[51]. The strongest supporting data were found employing in-situ hybridization and immunostaining to identify replicating SARS-CoV-2 RNA, S, and nucleocapsid proteins RNA within hepatocytes[51,52].Viral genomic RNA was also identified in postmortem COVID-19 Liver examinations[53,54], with observed intrahepatic presence of SARS-CoV-2 by EM and by in-situ hybridization [55-57], and viral replication within hepatocytes[58,59], thus reinforcing the role of direct SARS-CoV-2 hepatocyte injury. SARS-CoV-2 particles without membrane-bound vesicles were found in the hepatocyte cytoplasm of COVID-19 patients with aberrant LFTs[51], which is additional proof.

Liver hypoxia

Hypoxia can cause hepatocytes inflammatory cells infiltration, lipid accumulation, an increase in reactive oxygen species (ROS), and death[60,61]. ROS peroxidation products act as a second messenger amplifying the release of multiple cytokines[62]. In COVID-19, hypoxia and cytokine storm syndrome (CSS) are considered as risk factors for LFT abnormalities[63]. Hypoxic hepatitis features (*e.g.* centrilobular necrosis) are widely shown in postmortem liver biopsies[64]. In severe COVID-19, IMV, positive end-expiratory pressure, and/or vasopressor support negatively impact hepatic perfusion by lowering cardiac output, raising hepatic vascular resistance, and increasing portal vein pressure, which obstructs venous drainage, leading to acute LI (ALI) and/or cholestasis[58,65,66]. Gut ischemic injury on the other hand, results in intestinal endotoxinaemia and activation of the sympathetic nervous and adrenocortical systems furthermore contributing to LI[58,66]. Additionally, Kupffer cells can stimulate cytokines due to ischemia[67], while mitochondrial damage by SARS-CoV-2 results in aspartate aminotransferase (AST) release[68-70]. Direct interaction of mitochondrial proteins with the virus nonstructural protein 5 provides a probable reason for the AST-dominant liver profile[71]. In addition, unlike the hepatic preponderance of ALT, zone 3 of the hepatic acini containing higher AST concentrations is more susceptible to hypoxic injury[72]. In COVID-19 aminotransferases elevations, typically mild, are incompatible with very high AST/ALT elevations of primary hypoxic hepatitis[73]. Secondary hypoxic LI owing to the presence of acute respiratory distress syndrome (ARDS) as well as to an overactive inflammatory response to SARS-CoV-2 and MODS[73] might be implicated. In addition, cell iron overload might play a role, and hepcidinmimetic action of S protein may induce ferroportin blockage[25].

Cytokine storm syndrome

In order to maintain homeostasis, the body activates the immunological defense system and the oxidative stress response with the release of many cytokines when activated by endogenous or external stimuli like viruses[74]. Severe COVID-19 exhibits a distinct immunological dysregulation with two essential characteristics: Lymphocyte dysregulation with lymphopenia and overproduction of pro-inflammatory cytokines by monocytes[75,76]. The relationship between lymphopenia and CSS in COVID-19 pathogenesis was described in previous coronaviruses outbreaks[77,78]. Severe hypercy-



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Figure 1 Mechanisms implicated in COVID-19-induced liver injury. The different width of the red arrows represents the different contribution/significance of each separate mechanism in coronavirus disease 2019-associated liver injury. ACE2: Angiotensin converting enzyme-2; ADAMTS 13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif; Ang II: Angiotensin II; ARDS: Acute respiratory distress syndrome; CAM-1: Cell-adhension molecule-1; DIC: Disseminated intravascular coagulation; GM-CSF: Granulocyte-macrophage colony-stimulating factor; GSK: Conserved serine/threonine kinase; IL: Interleukin; JAK1: Janus kinase 1; LSECs: Liver sinusoidal endothelial cells; MAS: Macrophage activation syndrome; MPO: Myeloperoxidase; MCP-1: Monocyte chemotactic protein-1; NADPH: Nicotinamide adenine dinucleotide phosphate; NETS: Neutrophil extracellular traps; NRF2: Nuclear factor erythroid 2-related factor 2; NSAIDS: Non-steroidal anti-inflammatory drugs; PAI-1: Plasminogen activator inhibitor-1; PLT: Platelet; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of

transcription 3; TF: Tissue factor; TLR4: Toll-like receptor 4; TMPRSS2: Transmembrane serine protease-2; TNF-α: Tumor necrosis factor alpha; TPA: Tissue plasminogen activator; vWF: Von Willebrand factor; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

tokinemia results in a cascade of actions leading to tissue (especially liver) damage and MODS[79]. Lymphopenia, decreased CD4+, early and persistent elevation of cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-2, -6, -7, -10, -18, granulocyte-colony stimulating factor, interferon gamma (IFN-γ), interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory-protein-1a, chemokines], lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin, D-dimer and of coagulopathy markers (thrombopoietin), are independent risk factors for SLI (Table 1), and are linked to unfavorable outcomes[23,37,50,59,80-87]. CSS in severe COVID-19 is also associated with reduced CD8+, CD3+ and CD4+ T-cells[88,89]. Depletion of circulating CD8+ T-cells, the main determinant of LI in viral infections (influenza, measles, and SARS), reflects their trapping in the liver[90,91]. The syndrome known as "bystander hepatitis," which is frequently seen in systemic viral infections[92] and in COVID-19[93], is caused by circulating cytokines activating hepatic immune cells without compromising liver function. By activating Kupffer cells in the absence of viral antigens in the liver, viral-specific CD8+ T-cells that are confined to locations outside the liver may cause T-cell-mediated hepatitis[94]. Also, T-cells depletion cannot control the viral infection, leading to macrophage activation and more secondary inflammatory reactions[95,96]. In severe situations, the SARS-CoV-2 virus may cause a hyperinflammatory disease known as macrophage activation syndrome (MAS) or secondary hemophagocytic lymphohistiocytosis[75,76]. This syndrome is characterized by CSS, cytopenias, disseminated intravascular coagulation (DIC) and MODS. The pathogenesis of cytokine-driven hyperinflammatory disorders is heavily dependent on IL-6 signaling[76], that strongly correlates with elevated transaminases[88]. Inflammasome, a complex intracellular protein that SARS-CoV-2 may produce, helps promote caspase-1's autocatalytic activation (apoptosis/pyrolysis) and the exudation of pro-inflammatory cytokines[97], triggering the expression of other genes involved in the immune process[98], therefore resulting in MODS[87]. However, patients with mild COVID-19 may experience LFT abnormalities regardless of their inflammatory condition, probably because the unique inflammation brought on by SARS-CoV-2 is more likely to do so than inflammation brought on by other pathogens[99].

Endotheliopathy– hypercoagulable state– immuno-thromboinflammation

COVID-19 is considered to affect the endothelium, one of the largest organs in the human body[82]. SARS-CoV-2 may worsen microcirculation and encourage thrombus formation, tissue oedema, and organ ischemia by encouraging endothelial cell damage in the arteries, veins, arterioles, capillaries, and venules of all major organs[87,100-101]. Hepatic artery branches in the portal tract with endothelial enlargement and luminal constriction, as well as portal vein endophlebitis, and endotheliitis (leukocyte attachment to the vascular wall) with thrombotic material[102-105], are pathology findings indicative of endotheliopathy in COVID-19-related LI. The observed network of sinusoids decorated by CD34 suggests abnormal hepatic blood circulation[102]. In deceased patients with elevated ALT, significantly higher fibrinogen, factors VIII and II activity, and platelet marker CD61 liver staining was morphologically shown, in accordance with their serum levels (fibrinogen, D-dimer, von Willebrand factor (vWF) activity and antigen, and CRP[45,46,106,107]), thus resembling a microangiopathy thrombotic state[86,106,107]. Additionally, vWF-positive areas correlate with CD61-positive areas[60,101] and with intralobular neutrophil infiltration suggesting a link between the procoagulant state and liver inflammation[45,107]. Endotheliopathy, vWf expression on cell surfaces, and platelet adhesion are all mediated by IL-6 trans-signaling in liver sinusoidal endothelial cells (LSECs), which also plays a role in LSECs inflammation and activation of coagulation therefore being involved in COVID-19-related LI[45,107,108]. As LSECs are endothelial cells and do not express IL-6Ra, trans-signaling is thought to be the main method of IL-6 signaling to LSECs[109,110]. The Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway may also be used to promote IL-6 trans-signaling[45,107], which is essential for inducing a procoagulant and proinflammatory LSECs phenotype[111,112]. Activated neutrophils may generate neutrophil extracellular traps[113]. Decreased ADAMTS13 Levels, another typical finding in severe COVID-19 can induce increased platelet-endothelial interaction[111,114-118], while DIC may also result from CSS in critical/fatal COVID-19[119,120]. A significant imbalance between inhibitors and activators of fibrinolysis is also demonstrated. Reduced action of endogenous anticoagulants [antithrombin, tissue factor (TF) pathway inhibitor, and proteins C and S[121]] is a hallmark of hemostasis dysregulation. As the pulmonary inflammation worsens, hypofibrinolysis is caused by the consumption of plasminogen, high levels of plasminogen activator inhibitor-1 (PAI-1) and a decrease in tissue plasminogen activator, which prolongs the prothrombotic state[122,123].The pathological state involving platelet hyper-reactivity, hypercoagulability and hypofibrinolysis during COVID-19 is named “immuno-thromboinflammation”[123]. As platelets express both ACE2 and TMPRSS2 on their surface[124], it is intriguing that SARS-CoV-2 can attach to them directly and activate them, causing the release of clotting factors and inflammatory chemicals. Endothelial injury triggers the release of TF in circulation which may be also derived from macrophage/monocyte cells as a

Table 1 Liver function tests and factors associated significantly with clinical outcomes in COVID-19

Author/yr	Type of study (*n* of patients)	Factor	Outcome - statistical significance (severity/mechanical ventilation/ICU/mortality)
Krishnan *et al*[321], 2022, United States	Retrospective (*n* = 3830)	TBIL[1]	
		2–5 × ULN	Mortality risk significantly increased 6-fold ($P < 0.001$)
		> 5 × ULN	Mortality risk increased 7.86-fold ($P = 0.005$)
		AST[1]	
		2–5 × ULN	All-cause mortality HR, 1.49; $P < 0.001$
		> 5 × ULN	All-cause mortality HR, 2.19; $P = 0.005$
		ALP[1]	
		1–2 × ULN	All-cause mortality risk increased 1.42-fold ($P = 0.009$)
		> 2–5 × ULN	All-cause mortality risk increased 1.81-fold ($P = 0.032$)
		Inflammatory markers	
		CRP	aHR, 1.04 associated with mortality ($P = 0.001$)
		Ferritin	aHR, 1.0 associated with mortality ($P = 0.001$)
		IL-6	aHR, 1.0 associated with mortality ($P = 0.001$)
		neutrophil count	aHR, 1.0 associated with mortality ($P = 0.008$)
		D-Dimer	aHR, 1.03 associated with mortality ($P = 0.004$)
		LDH	aHR, 1.0 associated with mortality ($P < 0.001$)
		AST, ALT, TBIL	Significantly increased for those who received MV ($P < 0.0001$)
Kodavoor *et al*[180], 2022, India	Retrospective (*n* = 708)	AST[1]	aOR 1.007, per 1 IU/L increase for SD
		AST[1]	aHR 1.002 per 1 IU/L increase for mortality
		Sensitivity/specificity	90.6%/67% to predict mortality
		PPV/NPV	17.5%/95.73% to predict mortality
		Albumin[1]	aOR 0.217 per 1 g/dL increase for SD
			aHR 0.396 per 1 g/dL increase for mortality
Lombardi *et al*[230], 2022, Italy	Retrospective (*n* = 382)	Transaminases[1]	
		> 2 × ULN	OR 2.6, 95%CI: 1.3–6.7 for SD
		FIB-4 score < 1.45[1]	(OR 0.4; $P = 0.04$) protective factor for mortality
Hartl *et al*[326], 2022, Austria	Retrospective (*n* = 900)	AST[1]	aHR: 1.47; $P = 0.043$ for mortality
		TBIL[1]	aHR: 2.20; $P = 0.009$ for mortality
Siddiqui *et al*[229], 2022, United States	Retrospective (*n* = 1935)	Abnormal LFTs	
		Liver injury defined as: (AST/ALT > 3 × ULN or ALP/TBIL > 2 × ULN)	RR, 4.26; $P < 0.0001$ risk for mortality
		Mild elevated enzymes	RR, 5.52; $P < 0.0001$ for ICU admission
		(Levels lower than LI)	RR, 11.01; $P < 0.0001$ for MV
			RR, 2.16; $P < 0.0001$ for mortality
			RR, 2.48; $P < 0.0001$ ICU admission
		Cirrhotics	RR, 3.76; $P < 0.0001$ for MV

			RR, 2.19; P = 0.0022 for mortality
Cai *et al*[20], 2020, China	Retrospective (n = 417)	Hepatocellular LI	OR, 2.73; P = 0.02 for severe disease
		Mixed LI	OR, 4.44; P < 0.001 for severe disease
		LI[1]	aOR, 9.04; P < 0.001 for severe disease
Huang *et al*[191], 2020, China	Retrospective (n = 675)	AST[1] 3-fold ULN	aOR, 19.27; P < 0.0001 for mortality
			aOR, 116.72; P < 0.0001 for MV
Lei *et al*[192], 2020, China	Retrospective (n = 5771)	AST[1] 40-120 U/L	aOR, 4.81; P < 0.001 for all-cause mortality
		AST[1] > 120 U/L	aOR, 14.87; P < 0.001 for all-cause mortality
Ding *et al*[22], 2020, China	Retrospective (n = 2073)	Abnormal AST[1]	aHR, 1.39; P = 0.027 for mortality
		Abnormal DBIL[1]	aHR, 1.66; P = 0.001 for mortality
		LI during hospitalization[1]	aHR, 4.63; P < 0.001 for in-hospital mortality
		LI at admission[1]	aHR 1.87; P = 0.003 for in-hospital mortality
		Mixed LI[1]	aHR, 4.77; P < 0.001 for in-hospital mortality
		Cholestatic LI[1]	aHR, 3.99; P = 0.008 for in-hospital mortality
Phipps *et al*[23], 2020, United States	Retrospective (n = 3381)	Ferritin[1]	OR, 2.40; P < 0.001 for SLI
		IL-6[1]	OR, 1.45; P = 0.009 for SLI
		Peak ALT[1]	OR, 1.14; P = 0.044 for mortality
		Older age[1]	OR, 1.07; P < 0.001 for mortality
		DM[1]	OR, 1.30; P = 0.045 for mortality
Medetalibeyoglu *et al*[221], 2020, Turkey	Retrospective (n = 554)	AST/ALT > 1	AUC = 0.713, P = 0.001 marker of mortality risk
			AUC = 0.636, P = 0.001 for ICU admission
Chen *et al*[199], 2020, China	Retrospective (n = 502)	Grade of Liver damage[1]	aHR, 1.377; P = 0.049 risk factor for mortality
Mishra *et al*[200], 2021, United States	Retrospective (n = 348)	AST[1] (1 unit increase) IU/L Peak AST[1] (1 unit increase)	OR, 1.011; P = 0.006 for mortality
		Peak ALT[1] (1 unit increase)	OR, 1.007; P < 0.001 for mortality
		TBIL[1] (1 unit increase) mg/dL	OR, 1.005; P = 0.003 for mortality
		Alb[1] (1 unit increase) g/dL	OR, 1.997; P = 0.04
		Male[1]	OR, 0.5; P = 0.01
		BMI > 40 kg/m^2	OR, 1.94; P = 0.001
		LI[1]	OR, 2.17; P = 0.003
			OR, 1.79; P = 0.008
Chew *et al*[190], 2021, United States	Retrospective (n = 834)	Ischemic disease state[1]	OR, 2.4; P = 0.001 for mortality
		Hypecoagulable[1]	OR, 1.7; P = 0.02 for mortality
		Hyperinflammatory[1]	OR, 1.9; P = 0.02 for mortality
Ponziani *et al*[327], 2021, Italy	Retrospective (n = 515)	ALP[1] peak value	aOR, 1.007; P = 0.005 for mortality
		CRP[1]	aOR, 1.007; P = 0.008 for mortality
Piano *et al*[246], 2020, Italy	Retrospective (n = 565)	Abnormal LFTs[1]	OR, 3.53; P < 0.001 for ICU admission/death
Yip *et al*[287], 2021, China	Retrospective (n = 1040)	ALT/AST[1] ≥ 2 × ULN	aOR, 7.92; P < 0.001 for ICU/MV/death
Marjot *et al*[237], 2021, multinational	Retrospective (n = 785)	Age[1]	OR, 1.02; P = 0.011 for mortality
		Cirrhotics CTP-A[1]	OR, 1.90; P = 0.040 for mortality
		Cirrhotics CTP-B[1]	OR, 4.14; P < 0.001 for mortality

		Cirrhotics CTP-C[1]	OR, 9.32; $P < 0.001$ for mortality
		ArLD[1]	OR, 1.79; $P = 0.040$ for mortality
Lee *et al*[328], 2020, South Korea	Retrospective ($n = 1005$)	Age[1]	aHR = 4.96; $P < 0.001$ for mortality
		Liver cirrhosis[1]	aHR = 2.86; $P = 0.042$ fro mortality
		DM[1]	aHR = 2.29; $P < 0.001$ for mortality
		COPD[1]	aHR = 4.52; $P = 0.001$ for mortality
Singh *et al*[236], 2020, United States	Retrospective ($n = 2780$)	CLD[1]	RR, 2.8; $P < 0.001$ risk of mortality
		propensity matching	RR, 3.0; $P = 0.001$ risk of mortality
		Cirrhotics[1]	RR, 4.6; $P < 0.001$ risk of mortality
Hashemi *et al*[232], 2020, United States	Retrospective ($n = 363$)	CLD[1]	aOR 1.77; $P = 0.04$ for ICU admission
			aOR, 2.08; $P = 0.0092$ for IMV
		Cirrhotics[1]	aOR, 12.5; $P = 0.009$ mortality risk
Sarin *et al*[235], 2020, Asian	Retrospective ($n = 228$ CLD)	Cirrhotics[1]	
		AST/ALT > 1.4	HR = 1.4; $P = 0.02$ for mortality
		Obesity	OR = 8.1; $P = 0.002$ for LI
		Decompensated	OR = 2.5; $P = 0.05$ for mortality
		CTP score > 8	HR = 19.2; $P < 0.001$ for mortality
		DM in CLD non-cirrhotics	OR = 2.1; $P = 0.01$ for LI
Wang *et al*[51], 2020, China	Retrospective ($n = 657$)	Male gender[1]	OR, 2.038; $P < 0.001$ for LI
		hsCRP ≥ 10 mg/L	OR, 1.733; $p = 0.014$ for LI
		NLR ≥ 5	OR, 2.154; $P < 0.001$ for LI
Zhang *et al*[183], 2020, China	Retrospective ($n = 218$)	Male[1]	OR, 6.203; $P < 0.001$ risk for LI
		Neutrophil percentage[1]	OR, 1.004; $P = 0.003$ risk for LI
		CRP[1]	$P < 0.001$ in LI patients
		D-dimer[1]	OR, 1.486; $P < 0.001$ risk for LI
Shauly-Aharonov *et al*[329], 2021, Israel	Retrospective ($n = 37121$)	Age	OR = 1.1 for every year increase; $P < 0.001$) risk for severity
		Male gender	OR = 1.34; $P = 0.012$ risk for severity
		BMI	OR = 1.02 for 1 kg/m^2 increase; $P = 0.025$ risk for severity
Kovalic *et al*[208], 2020, United States	Meta-analysis ($n = 24299$)	CLD[1]	Pooled OR, 1.48; $P = 0.001$ for severity
			Pooled OR, 1.78; $P = 0.02$ for mortality
Kulkarni *et al*[6], 2020, India	Meta-analysis Multinational ($n = 20874$)	Increased LFTs	OR, 3.46; $P < 0.001$ for mortality
			OR, 2.87; $P < 0.001$ for severe disease
Sharma *et al*[207], 2021, United States	Meta-analysis ($n = 12882$)	AST[1]	Pooled OR, 2.98; $P < 0.00001$ for poor outcomes
		ALT[1]	Pooled OR, 1.73; $P < 0.0001$ for poor outcomes
Del Zompo *et al*[323], 2020, Italy	Meta-analysis ($n = 20724$)	ALT[1]	OR 1.54, 95%CI: 1.17-2.03 for severity
		ALT[1]	OR 1.48, 95%CI: 1.12-1.96 for mortality
		AST[1]	OR 3.17, 95%CI: 2.10-4.77 for severity
		AST[1]	OR 4.39, 95%CI: 2.68-7.18 for mortality
		TBIL[1]	OR 2.32, 95%CI: 1.18-4.58 for severity

	TBIL[1]	OR 7.75, 95%CI: 2.28-26.40 for mortality

[1]Age, gender, ethnicity, race, BMI, and all the pre-existing comorbidities were adjusted as confounders.

Results come from multivariate analysis and logistic regression (studies with more than 200 individuals are included). aHR: Adjusted hazard ratio; ALB: Albumin; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; aOR: Adjusted odds ratio; ArLD: Alcohol related liver disease; AST: Aspartate transaminase; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; CRP: C-reactive protein; CTP: Child-Turcotte-Pugh; CLD: Chronic liver disease; DM: Diabetes mellitus; FIB-4: Risk of fibrosis score; GGT: Gamma-glutamyl transferase; HR: Hazard ratio; ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LDH: Lactate dehydrogenase; LI: Liver injury; LFTs: Liver function tests; NLR: Neutrophil-to-lymphocyte ratio; NPV: Negative predictive value OR: Odds ratio; RR: Relative risk; PPV: Positive predictive value; SLI: Severe liver injury; MOF: Multiple organ failure; TBIL: Total bilirubin; ULN: Upper limit of normal.

consequence of MAS[125]. When PAI-1 is overproduced, it binds to TLR4 on macrophages and triggers the release of cytokines and chemokines[126], which in turn promotes inflammation, steatosis, and microvascular thrombosis[127]. SARS-CoV-2 binds to the ACE2 receptor on tissues inceasing Ang II levels, favoring PAI-1 and TF expression thus promoting hypercoagulability and impairing fibrinolysis [115]. Extensive pericyte activation during LI contributes to the recruitment of inflammatory cells, and their conversion into cells that resemble myofibroblasts results in the creation of extracellular matrix proteins and the ensuing fibrosis of the vessel wall[128].

Oxidative stress

The expression of antioxidant proteins is regulated by nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that is triggered by oxidative stress[129,130,131]. One of the most significant mechanisms of antioxidant and anti-inflammatory element signaling is the complex Kelch-like ECH-associated protein 1-NRF2-antioxidant response element[132]. The NRF2 antioxidant pathway is suppressed in COVID-19, while infected cells show a GSK-3 (conserved serine/threonine kinase) activity that degrades NRF2[133]. NRF2 interacts with NF-kB, a proinflammatory signal transduction pathway[134] driving the initial proinflammatory response[135], to reciprocally regulate redox metabolism[136-139]. When NRF2 activity reaches its maximum level, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation is inhibited. In response to viral infection or other stimuli, inhibitor kappa B (IB) is phosphorylated, which releases and translocates NF-B to the nucleus, causing inflammatory cascades and the generation of inflammatory mediators[140]. *Via* the TNFR1-NF-B signaling axis, TNF- may activate NF-B[141], and NF-kB in turn enhances the release of inflammatory cytokines[135]. This creates a vicious loop that feeds CSS and exacerbates LI[142-144]. Oxidative stress is mostly caused by ROS. The NF-B pathway is activated by COVID-19, ARDS, and sepsis as they cause tissue ischemia and ROS production[145]. In the early stages of COVID-19, ACE2 is the most critical component, whereas the IL-6-STAT3 axis is crucial in the late stages and in CSS[146]. Indeed, both NF-κB and STAT3 pathways are activated in COVID-19 promoting inflammation by activating the IL-6 amplifier[147]. NRF2 promotes Glutathione synthesis[148], and participates in the tricarboxylic acid cycle by regulating the production of NADPH, a key co-factor of antioxidant reactions[149,150]. NRF2 inhibits liver fibrosis and promotes liver regeneration[151-153] therefore protecting liver cells in viral hepatitis[154], drug-induced LI (DILI)[155-157], cholestasis[144,158,159], and NAFLD[160,161], by reducing gluconeogenesis and fat deposition, restoring insulin resistance, and boosting the anti-inflammatory and antioxidant effects[162].

Drug-induced liver injury

The large use of antiviral drugs may contribute to COVID-19-related LI especially in individuals with increased baseline ALT[163]. The pooled incidence of DILI in COVID-19 is reported 25.4%[6]. In DILI, AST usually peaks before ALT, a biochemical pattern also observed in severe COVID-19. In some cases, observed microvascular steatosis and mild hepatic inflammation are consistent with DILI[20,49]. In remdesivir-treated patients, 23%-35% show increased LFTs[164,165] indicating hepatotoxicity, while 2%–3% required treatment discontinuation[164]. Lopinavir/ritonavir incidence of DILI is 37.2%[9] with a significant increased risk (OR = 4.44) for severity[20]. Medicines with possible antiviral effects should only be administered on patients who have risk factors for severe illness[82] and early in the course of the disease[166]. In tocilizumab-treated patients, 15%-51% presented a transitory but not significant hypertransaminasemia between 9-13 d, some of which showed surprisingly higher mortality[167,168]. The liver's IL-6-mediated endotheliopathy should be improved by treatment with the JAK inhibitor baricitinib. A significant disadvantage of all those treatments those treatment clinical trials is the frequent exclusion of patients with AST/ALT > 5 × ULN[169]. Moreover, immunosuppressive drugs, such as tocilizumab, tofacitinib, and dexamethasone, can potentially induce LI *via* HBV reactivation in patients with occult infections[170,171], therefore antiviral prophylaxis should be administered. Dexamethasone may ameliorate endothelial injury[172] by dampening of endothelial IL-6 production [173]. The most typical contributors to DILI in the general population, antibiotics and nonsteroidal anti-inflammatory medications, may also cause LI, while acetaminophen can cause alterations in aminotransferases even at therapeutic doses[174].

Gut microbiome

In COVID-19, dysbiosis of the gut microbiota may have a significant impact on the clinical outcome of patients with comorbid conditions such diabetes, hypertension, and obesity and may lead to liver damage[175,176]. For example, older people often have less variety in their gut microbiota, and COVID-19 is more severe in this age group, supporting the possibility that microbes play a role in outcomes [177]. Hepatic dysfunction brought on by sepsis may result from disruption of the gut microbiota and a breach of the gut-mucosal barrier[178]. Moreover, the diversity of the gut microbiota influences how the host immune system responds[178]. It is hypothesized that changes in the gut-liver axis may contribute to the severity of COVID-19 seen in cirrhotics. Cirrhosis is characterized by changes to intestinal permeability, gut microbiota composition, and function[179].

INCIDENCE

The earliest available epidemiological data of COVID-19 patients came from China. Abnormal LFTs were first reported in a cohort from Wuhan, China[9], making liver the most frequently damaged outside of the respiratory system. It's interesting to note that Wuhan, the COVID-19 epicenter, had a substantially greater incidence of elevated aminotransferases than the surrounding areas (21% *vs* 10%) [18,63] possibly because of higher SARS-CoV-2 doses exposure in Wuhan[80]. Western populations show abnormal LFTs more clearly than Eastern populations (Figure 2). The timing of LFT determination during disease course, different definitions, but mostly, the geographical variability in the prevalence of underlying diseases are the determinants for the observed discrepancies[18]. With respect to worldwide published data the overall prevalence of abnormal LFTs ranges from 2.5% to 96.8% (Table 2), while SLI accounts for 4.94%-21.8% of COVID-19 patients[7,20,22,23,38,80,180]. Patients with SLI are younger and more likely to be male[23]. Younger patients may exhibit a more robust immune response to infection, causing LI and determining its degree[23,38]. Aminotransferases are higher in severe COVID-19 cases, in accordance with the 2002-2004 SARS outbreak[181]. Concerning cholestatic enzymes, elevated GGT and ALP range between 15%-47.3% and 4%-58.5% respectively (Table 2). GGT, a surrogate marker for increased oxidative stress and chronic inflammation[182], usually increases in severe cases[58,59] implying cholangiocyte injury[183,184]. The GGT elevation without accompanied by ALP elevation[59] may also develop in DILI more frequently than obstruction. ALP elevation is rare, usually < 2 × ULN [185], and is mostly observed in MODS or death from COVID-19[186]. The joint trajectory of GGT, ALP, and bilirubin points towards a cholestatic LI seen in impaired survival[187]. The prevalence of total bilirubin (TBIL) elevations ranges between 3.1% and 52.1%. Concerning longitudinal changes, LFTs become more frequently, and more severely deranged during hospitalization[20,188,189]. The median time to peak AST levels is 3 d after admission, normalizing within 4.4 d[190]. ALT elevations peak between 4-17 hospital day[188]. In deceased patients, ALT levels are normal in the first week but subsequently rise rapidly along with AST at the third week. In survivors, slightly elevated ALT levels occur at 2-3 wk after symptoms onset when AST levels might remain normal[191]. A biphasic pattern with early aminotransferase onset, culminating around days 10-15 of hospitalization, and then gradual normalization accompanied by rising ALP is also suggested[187]. ALI (ALT > 3 × ULN) occurs between 17-18.5 d after symptoms onset[28,192]. AST is diffusely represented in many tissues while ALT is considered liver-specific[193]. Greater AST levels may be related to mitochondrial damage or damage to other organs[194]. In the liver, ALT is only found in the cellular cytoplasm[72,195] whereas AST is both cytosolic (20%) and mitochondrial (80%) localized, and is in higher concentrations in zone 3 of the hepatic acinus therefore ischemic or toxic damage to this zone may result in greater AST elevations.

UNDERLYING DISEASES, SEVERITY, PROGNOSIS

Underlying diseases

The median age of COVID-19-induced LI patients ranges between 51.5-56 years with male predominance[65,191]. The incidence of hypertension, diabetes, and coronary heart disease ranges between 23.08%-31,8%, 11.54%-15.3%, and 7.8%-11.54%, respectively[192]. Age, male sex, hypertension, and diabetes are negatively correlated with SLI in COVID-19[23]. The association of LI with hypertension and poorer prognosis is more significant in the absence of pre-existing CLD[38]. CLD prevalence varies widely with Chinese studies being reported between 1.4%-15.3%[9,184,196], lower than in Western countries (5%-37.6%)[197,198]).

Severity

Risks of severity for specific LFTs indices are shown in Figure 3. Aminotransferases levels > 5 × ULN correlate to mortality[23,192], while incidence of LI is higher in ICU than non-ICU patients (61.5% *vs* 25.0%)[199]. Elevated LFTs on admission show a 3-fold greater risk of severe disease and 3.5-fold risk for mortality[6,200]. After adjustment, patients with LI are at a 9-fold greater risk of severe COVID-19

Table 2 Incidence of abnormal liver function tests (liver injury)

Author/citation LFTs performed	Type of study (*n* = participants)	Incidence (%)	Country/year of publication
Cai *et al*[20]	Study (*n* = 417)		China/2020
Abnormal LFTs			
SLI (AST/ALT > 3 × ULN		76.3	
or ALP/γGT > 2 × ULN)		21.8	
ALT (> 3 × ULN)		37	
GGT (> 3 × ULN)		41	
AST (> 3 × ULN)		20	
TBIL (> 3 × ULN)		10	
MOF		23.3	
Phipps *et al*[23]	Study (*n* = 2273)		United States/2020
Mild (peak ALT < 2 × ULN)		45	
Moderate (peak ALT 2-5 × ULN)		21	
SLI (peak ALT > 5 × ULN)		6.4	
Huang *et al*[191]	Study (*n* = 675)		China/2020
Abnormal LFTs		37.5	
SLI		7.7	
Guan *et al*[284]	Study (*n* = 1099)		China/2020
AST/ALT			
mild disease		18.2–19.8	
severe disease		28.1–39.4	
Hundt *et al*[185]	Study (*n* = 1827)		United States/2020
LFTs (on admission)			
AST		66.9	
ALT		41.6	
TBIL		4.3	
ALP		13.5	
Wang *et al*[65]	Study (*n* = 657)		China/2020
Liver injury		46.1	
ALT		42.2	
GGT		24.4	
TBIL		4.9	
Chu *et al*[320]	Study (*n* = 838)		China/2020
Liver Injury		51.2	
Yip *et al*[287]	Study (*n* = 1040)		China/2021
Aminotransferases		22.5	
ALP		58.5	
TBIL		52.1	
Ding *et al*[22]	Study (*n* = 2073)		China/2021
Survivors		90.3	
Any abnormal LFT		61.8	
Mild abnormal LFT		47.5	

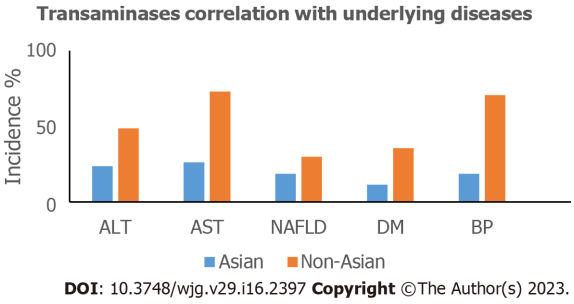
SLI		14.3	
LI type			
Hepatocellular		25.8	
Cholestatic		6.7	
Mixed		25.7	
Specific liver indices			
ALT		43.3	
AST		38.9	
GGT		31.8	
Shao *et al*[38]	Study (n = 1520)		China/2021
SLI		17.9	
Mishra *et al*[200]	Study (n = 348)		United States/2021
New-onset LI		52.8	
Sikkema *et al*[204]	Study (n = 382)		Netherlands/2021
LI		41.6	
Moderate LI (ALT > 100 or ALP > 200)		6.5	
Cholestatic LI		9.2	
Chew *et al*[190]	Study (n = 834)		United States/2021
AST		62.5	
ALT		33.7	
ALP		11.9	
TBIL		3.1	
Richardson *et al*[25]	Study (n = 5700)		United States/2020
AST		58.4	
ALT		39	
Bernal-Monterde *et al*[187]	Study (n = 540)		Spain/2020
Abnormal LFTs		64.3	
ALT		28.6	
AST		40.9	
GGT		47.3	
Krishnan *et al*[321]	Study (n = 3830)		United States/2022
ALT		70.4	
AST		44.4	
ALP		16.1	
TBIL		5.9	
Kodavoor *et al*[180]	Study (n = 708)		India/2022
AST		69.91	
< 1–2 times ULN		42.51	
2–3 times ULN		14.26	
3–5 times ULN		8.19	
> 5		4.94	
ALT		80.22	
< 1–2 times ULN		42.93	

2–3 times ULN		17.93	
3–5 times ULN		12.14	
> 5		7.2	
Russo *et al*[234]	Study (*n* = 1641)		Italy/2022
AST		27.7	
ALT		23	
TBIL		12.6	
Marjot *et al*[44]	Review		United Kingdom/2021
AST		29–39	
ALT		38–63	
Cai *et al*[82]	Review		China/2021
ALT		11–56.3	
AST		15–86.8	
TBIL		2.7–30.6	
CLD		2–11	
Ekpanyapong *et al*[322]	Review		Multinational/2022
Aminotransferases		10–58	
ALP		1–10	
TBIL		3–23	
GGT		13–54	
Esteban *et al*[209]	Review		United States/2022
Aminoransferases (admission)		20–67	
Aminoransferases (hospitalization)		61–83	
ALP		23–30	
TBIL		4–16	
Garrido *et al*[59]	Review		Portugal/2020
ALT		2.5–50	
AST		2.5–61.1	
TBIL		0–35.3	
Kullar *et al*[2]	Meta-analysis (*n* = 3046)		United States/2020
ALT		21	
AST		24	
TBIL		9	
Wijarnpreecha *et al*[198]	Meta-analysis (*n* = 64 studies) (*n* = 11245 pts)		United States/2021
AST		23.2	
ALT		21.2	
TBil		9.7	
GGT		15	
ALP		4	
AST			
Severe cases		45.5	
Non-severe		15	

Wu *et al*[253]	Meta-analysis (*n* = 45 studies)		Multinational/2018
Admission			
Any abnormal LFT		27.2	
ALT		20.4	
AST		21.8	
ALP		4.7	
GGT		35.8	
TBIL		8.8	
Hospitalization			
Any abnormal LFT		36	
ALT		38.4	
AST		28.1	
TBIL		23.2	
Del Zompo *et al*[323]	Meta-analysis (*n* = 36 studies) (*n* = 20724 patients)		Italy/2020
At admission (pooled prevalence)			
Abnormal LFT		46.9	
ALT		22.8	
AST		26.5	
GGT		22.5	
ALP		5.7	
TBIL		8	
Zhu *et al*[262]	Meta-analysis (*n* = 38 studies) (*n* = 3063 pts)		China/2020
Abnormal LFTs		29	
Mao *et al*[18]	Meta-analysis (*n* = 1267)		China/2020
Abnormal LFTs		19	
Alqahtani *et al*[324]	Meta-analysis (*n* = 30 studies)		Multinational/2020
Abnormal LFTs		61.1	
Sultan *et al*[325]	Meta-Analysis (*n* = 47 studies) (*n* = 10,890 pts)		United States/2020
Pooled prevalence			
ALT		15	
AST		15	
TBIL		16.7	
Kumar *et al*[210]	Meta-analysis (*n* = 128 studies)		India/2020
Pooled prevalence			
TBIL		13.71	
ALT		31.1	
AST		33.95	
ALP		6.99	
GGT		30.62	
ALB		61.57	
Severe *vs* non-severe pts			

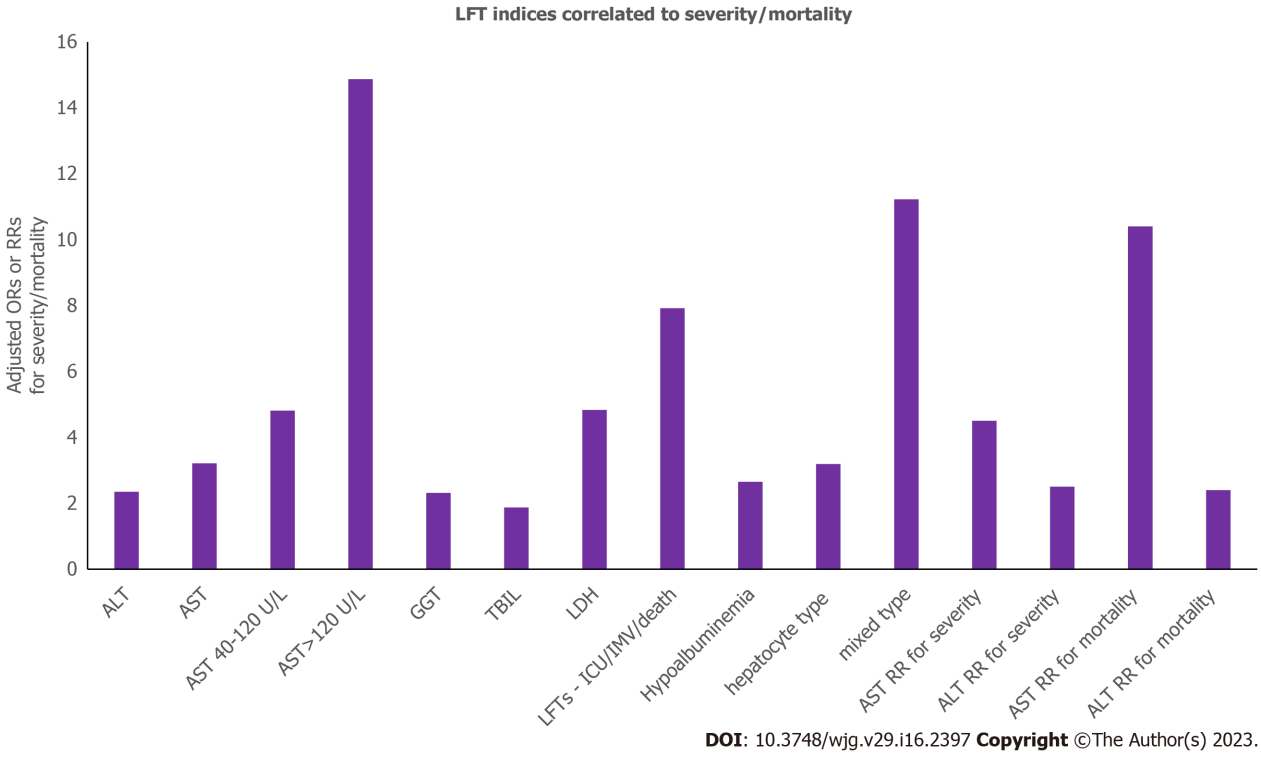
TBIL	18.80 *vs* 9.24
ALT	39.58 *vs* 24.15
AST	49.68 *vs* 19.40
ALP	11.33 *vs* 4.0
GGT	46.90 *vs* 18.66
ALB	75.91 *vs* 31.04

The table encompasses several studies and meta-analyses which included more than 200 individuals.
ALB: Albumin; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transferase; LI: Liver injury; LFTs: Liver function tests; SLI: Severe liver injury; MOF: Multiple organ failure; TBIL: Total bilirubin; ULN: Upper limit of normal; γGT: Gamma-glutamyltransferase; LFT: Liver function tests; pts: Patients.



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Figure 2 Transaminases correlation with underline disease in Asian and non-Asian COVID-19 populations. BP: Blood pressure; DM: Diabetes mellitus; NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019.



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Figure 3 Adjusted liver function test indices and type of liver injury correlated to COVID-19 severity and mortality. ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LFTs: Liver function tests; OR: Odds ratio; RR: Relative risk; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase LDH: Lactate dehydrogenase; TBIL: Total bilirubin; COVID-19: Coronavirus disease 2019.

[20], and at a 7.5-fold risk for mortality[201]. A few studies however, failed to show such an association with severity/mortality[202-205], disease progression[17], and ICU admission[202,206]. These differences could be attributed to the nature of the studies that were included and the various outcomes definitions[207,208]. It seems that LI portends the need for ICU care and IMV[209]. CLD is also associated with severity [odds ratio (OR)/relative risk (RR) = 1.48, 1.70][208,210-213], and mortality (OR/RR = 1.08, 2.65)[208,212,214] while in a few studies such an association was not observed[6,207,215-219] (Table 1). Age, male gender, higher body mass index (BMI), corticosteroids, antifungals, lymphocyte decrease, neutrophil increase, and CLD are factors positively associated with ALT/AST increase[6,18,20,192,194,220]. ALP levels are tightly associated with male gender, antifungals, neutrophil count increase, and CLD. Antifungals, antivirals, systemic corticosteroids, and platelet count reduction are positively correlated with increased TBIL levels.

Within five weeks, patients with SLI are significantly more likely to have been intubated, to require renal replacement therapy, or to decease compared to moderate/mild LI[23]. LFTs elevations during hospitalization correlate well with the severity of inflammatory indexes (CRP, procalcitonin, ferritin, LDH, GGT, lactate, and D-dimer)[16,200,221]. In essence, LFTs can be used as a surrogate for the monitoring of inflammation. ALT levels correlate well with the CSS inflammatory markers[317,222-225] (Table 3). This immunological response is consistent with that reported for other viral respiratory tract infections[226]. IL-6, -8, TNF-α are positively correlated with the increased AST, TBIL, and ALP, therefore, cytokines contribute to COVID-19-induced LI[65]. Significantly higher white blood cell (WBC) and neutrophils, and lowered lymphocytes are observed in LI[65,227,228].

Prognostic factors

In mixed-effect Cox model adjusted for age, gender, and comorbidities patients with AST > 3 × ULN compared to normal AST exhibited increased risks of death and IMV (19.27-fold and 116.72-fold, respectively)[191]. Risk of severity and all-cause mortality of LFTs abnormalities are shown in Figure 3. Patients with LI have a 4-fold higher rate of mortality, 7-fold higher rate of ICU admission, and 11-fold higher rate of intubation[20,229], while hepatocellular and cholestatic type LI increases the risk by 3-fold [20]. LI is suggested as an independent prognostic factor of COVID-19[200,211]. AST/ALT ratio > 1 predicts mortality, severe pneumonia and ICU admission[221]. While hepatic steatosis is considered to have no impact on disease course, fibrosis (FIB-4) score < 1.45 is a significant protective factor[230]. Vasopressor use (ischemia), and hyperinflammatory/hypercoagulable state are also independent predictors of death[190]. ALP peak value is a risk factor for in-hospital mortality[231]. All-cause adjusted mortality risk is 6-fold significantly increased in patients with an elevated TBIL > 2-5 × ULN and 1.42-fold in patients with ALP > 1-2 × ULN[232]. Serum albumin is negatively associated with severity. Hyperglycaemia at admission is associated with severity/mortality[233]. In fully models adjusted for confounders, increasing age, non-white and non-black race, hypertension, overweight/obesity, kidney disease, cardiovascular disease, diabetes, cancer, and dementia, are independently associated with an increased risk of in-hospital mortality[232-234]. Higher state of inflammation is also significantly associated with mortality[232], while peak ferritin and IL-6 Levels are associated with SLI (Table 1)[23].

COVID-19 IN DIFFERENT CLD POPULATIONS

Cirrhosis

In COVID-19 the presence of cirrhosis, mostly of decompensated, is an independent predictor of liver-related (OR = 3.24) and overall complications, as well as mortality (aOR; 11.3-12.5)[232,235]. In patients with CLD, the 30-d cumulative overall mortality is higher in cirrhotics (RR = 4.6)[236], with respiratory complications being the main cause of death[208,237-239].In terms of CLD etiology, more frequent is viral (60.5%), followed by NAFLD (32.6%), alcohol-related liver disease (ArLD) (4.7%), and autoimmune hepatitis (2.3%). ArLD, NAFLD and hepatocellular carcinoma (HCC) but neither viral nor autoimmune hepatitis are associated with increased mortality[237,240-242]. Cirrhosis-associated immune dysfunction (CAID), is a condition that affects patients with CLD, particularly cirrhosis, who display a variety of immune dysfunctional mechanisms that enhance their vulnerability to infection and abnormal inflammatory responses[44]. The CAID phenotypes represent a continuum of dynamic events that shift from being primarily pro-inflammatory to being primarily immunodeficient. Reduced bacterial opsonization, phagocytosis, protein C activity, antigen T-lymphocyte dependent responses, vaccination responses, hypoalbuminemia, hypocomplementaemia, and intestinal dysbiosis are some of the characteristics of this condition[44,243,244]. CAID is also associated with increased serum levels of IL-1β, -6, -17, -18, TNF-α, and IFN-γ[245], and predisposes to a variety of viral or fungal-related diseases[246]. Despite the increased risk of infection[247], cirrhotics exhibit a lower risk of acquiring SARS-CoV-2[209,248,249], whereas in large population studies patients with CLD are not over-represented[250]. In patients with CLD, a condition known as acute-on-chronic liver failure (ACLF) is characterized by abrupt hepatic decompensation and extrahepatic organ failures and is linked with a significant short-term mortality [251]. While being typically linked to bacterial infections, COVID-19, among other viruses may cause

Table 3 Serum parameters alone or in combination associated with specific outcomes

Parameters	Associated conditions
ALT	CSS inflammatory markers
Elevated serum IL-2R, IL-6, TNF-α	LI
IL-6, ferritin, CRP, ESR, Procalcitonin, hypoalbuminemia, low PLTs, low CD4+ T-cells and B-lymphocytes	Non-favorable course of LI
Simultaneous increase in IL-6 + ferritin + ALT + hypoalbuminaemia	Significant LI
On admission increased inflammatory markers + AST + GGT + LDH + lymphopenia+eosinopenia	More Severe clinical course
Lymphopenia, Thrombocytopenia	Disease severity
Thrombocytopenia	Consumptive coagulopathy
Low Hb	Controversial data

ALI: Acute liver injury; ALT: Alanine transaminase; AST: Aspartate transaminase; CRP: C-reactive protein; GGT: Gamma-glutamyl transferase; LDH: Lactate dehydrogenase; LI: Liver injury; CSS: Cytokine storm syndrome; IL: Interleukin; ESR: Equivalent series resistance; TNF-α: Tumor necrosis factor-α; PLT: Platelet; Hb: Hemoglobin.

ACLF[251-253].The incidence of ACLF in COVID-19 patients with CLD ranges from 10% to 50%[235,237], with a reported 65% mortality rate[237]. ACLF may also occur in compensated cirrhotics with severe COVID-19[254]. Also, as the severity of cirrhosis is assessed by the Child-Turcotte-Pugh (CTP) score, there is a stepwise rise in morbidity and mortality[237]. After adjusting for baseline characteristics, COVID-19-related mortality is significant across the CTP stages (aORs): A = 1.90, B = 4.14 and C = 9.32[237], similar to non-COVID-19 hospitalized cirrhotics[235,238]. CTP score > 9 at presentation independently predicts mortality, in accordance with MELD and chronic liver failure consortium scores [235,238,239]. In cirrhotics LI is evident at presentation (OR = 6.2)[200]. The non-survivors cirrhotics have higher AST levels, AST/ALT ratio > 1.4 (aHR = 1.4), and a low R value that predicts mortality [235]. Development of liver decompensation during COVID-19 exhibits increased mortality compared to maintenance of hepatic compensation (63.2% *vs* 26.2%)[239].

Despite suffering higher mortality, those cirrhotics who survive the initial insult show re-admission/death rates at 90-d comparable to cirrhotics without COVID-19[254]. SARS-CoV-2 does not appear to accelerate the progression of liver disease beyond cirrhosis' normal course after acute infection. The interaction of severe COVID-19, pulmonary illness, and ACLF is probably mediated by CAID. Cirrhosis is linked to an increase in cytokine production and baseline endotoxinaemia, which may cause an increased inflammatory response to infection[44]. Hypercytokinemia, another characteristic of COVID-19, causes hepatocyte apoptosis and necrosis, which, in the presence of depleted liver reserves, may result in hepatic decompensation[244,255]. Therefore, cirrhosis and COVID-19 may have detrimental effects on each other. Cirrhosis decompensation in COVID-19 is characterized by deteriorating ascites, worsening of jaundice and hepatic encephalopathy more frequent than variceal bleeding or spontaneous bacterial peritonitis[235,240,241]. Despite compensated cirrhotics having greater problems, mortality among them and non-cirrhotics is comparable, supporting the idea that there is adequate hepatic reserve for recovery. The more significant predictor of death in COVID-19[237] is hepatic decompensation as opposed to cirrhosis *per se*. The data indicate that non-traditionally hepatotropic infections, such as SARS-CoV-2, may directly precipitate ACLF in cirrhotic patients[256] as also observed in influenza[252]. The median platelet (PLT) count and IFN-γ are significantly decreased in CLD than non-CLD patients[226,257], and cirrhotics are at higher risk for thrombotic events[105].

Non-alcoholic fatty liver disease

NAFLD has been advocated as a risk factor for severe COVID-19, it is present in the majority of COVID-19 patients with CLD worldwide, and shows longer viral shedding time[258]. Patients with NAFLD assessed by CT scan and with intermediate/high risk of FIB-4 have higher risk for severe COVID-19 (OR = 2.95) *vs* patients without NAFLD fibrosis score (NFS), suggesting a pathogenetic role for advanced liver fibrosis in severe COVID-19 with worse outcomes[232,250-260]. Increasing FIB-4 or NFS values when included as continuous measures in multivariable regression, they are significantly associated with COVID-19 severity (aORs = 1.90, and 2.57, respectively). NAFLD is epidemiologically associated with an increased risk of severe COVID-19[261,262], independently of BMI[263]. Hepatic steatosis is an interesting pathological characteristic that frequently occurs in COVID-19. It is possible that activation of coagulation, which is capable of causing hepatic steatosis in NAFLD, represents a unique mechanism connecting thrombosis and steatosis, two diseases that are both common in COVID-19[264]. Given that high PAI-1 has been linked to NAFLD and NASH[265,266], the involvement of PAI-1 in COVID-19 may be noteworthy. Lastly, obesity, a major risk factor for thrombosis owing to adipocy-

tokine-mediated processes, increases inflammatory molecules, Ang II/Ang 1,7 imbalance, ROS-mediated endothelial dysfunction, and alterations in lipid/glucose metabolism[267-269]. Greater risk of severe COVID-19 is found in non-diabetics patients < 60 years with NAFLD (aOR = 4.07)[259,270]. Additionally, underlying diabetes with NAFLD shows a 2-fold higher risk of severe COVID-19, much higher when LI is present (OR = 6.4), or in obese NAFLD patients (aOR = 6.32)[271]. NAFLD is associated with an increased risk of severity when it coexists with obesity[272], in non-diabetics[270], in younger patients[259], and in individuals with high hepatic fibrosis scores[260]. Although it is unknown how obesity and NAFLD might worsen COVID-19, comparable mechanisms are frequently present in both disorders comprising alterations in the immune response, macrophage activation, and (low-grade) inflammation[260,273,274]. Obesity-related chronic inflammation impairs macrophage activation *via* antigen presentation and pro-inflammatory cytokine synthesis[275]. Moreover, obesity reduces B- and T-cell responses, which leads to greater susceptibility and delayed clearance of viral infections[275,276]. The advancement of COVID-19 is favored in NAFLD patients when hepatic macrophage polarization changes from M1 (which promotes inflammation) to M2 (which suppresses inflammation)[58,274,277]. The polarization states of macrophages may be unbalanced, influencing the host's inflammatory or tolerance response to SARS-CoV-2 signals provided *via* the gut-liver axis. ACE2 expression is elevated in CLD/NASH patients, and cytokine secretion is enhanced in association with COVID-19[42]. Conversely, in a few studies NASH was not associated with severe disease[278]. It is also demonstrated that COVID-19 patients exhibit increased serum levels of MCP-1, which exacerbates steatohepatitis [279]. Age, gender, obesity, and other comorbidities are thought to be less important risk factors for COVID-19 than NAFLD. NAFLD progression may also be hastened by COVID-19.

Alcohol-related liver disease

The secure-cirrhosis and COVID-Hep registries identified ArLD as a risk factor for COVID-19 mortality (aOR = 1.79) related to advanced disease and CAID[237]. Increased mortality is also seen in alcohol-related cirrhosis[238,239]. Immune dysregulation, particularly changes in the gut-liver axis, is accentuated in ArLD, with increased endotoxinaemia and Kupffer cell activation leading to proinflammatory cytokine transcription and superoxide production[44,178]. Moreover, alcohol impairs adaptive immunity, including both cell-mediated and humoral responses[179]. The inflammatory state caused by danger-associated molecular patterns is linked to ArLD leading to the production of pro-inflammatory cytokines[238,256]. It is postulated that CSS could aggravate the increased inflammatory process in ArLD, resulting in worse outcomes[119]. In the COVID-19 period, hospitalized patients with ArLD appear at more advanced disease stages, with acute decompensation, higher MELD scores, and greater rates of ICU and mortality[185].

Autoimmune hepatitits

Immunosuppressed patients exhibit higher SARS-CoV-2 viral titres, prolonged viral shedding but do not exhibit increased risk of complications[241-242,280,281]. Analysis of AIH *vs* non-CLD patients demonstrates increased risk of hospitalization, but equivalent risk of all other outcomes including death [281]. As a result, in stable patients, immunosuppression should not be lowered as a COVID-19 risk reduction strategy[11,282]. Immunosuppression may also reduce the risk of new-onset LI[242,281,282].

Viral hepatitis

Persons with chronic HBV or HCV infection who do not have cirrhosis are not more likely to get COVID-19 or have a worse outcome[9,59]. Conversely, patients with SARS-CoV-2 and HBV co-infection are prone to a worse prognosis[283] and tend to have 2.2-fold more severe COVID-19[284]. The in-hospital mortality in COVID-19 patients with HBV is 6.0%[22] with ACLF precipitation < 1%, while LI prevalence in non-decompensated is comparable with non-CLD patients[22]. Significantly lower monocyte and WBC counts, higher levels of CD8+ T-cells and thrombocytopenia in HBV with COVID-19 are observed compared to COVID-19 alone[285]. The likelihood of HBV reactivation during SARS-CoV-2 infection is mentioned, however the risk appears to be low[286]. Those with HCV who have COVID-19 are more susceptible to hospitalization, but not at a higher risk of death[287]. Because recently treated HCV patients were less prone to contracting with SARS-CoV-2, HCV antibodies may be indicative of "protection" against COVID-19[288].

Liver transplantation

Liver transplant (LT) recipients are more frequently diagnosed with COVID-19 than general population. This might be attributable in part to more careful surveillance and a lower viral testing criterion in LT recipients[289]. The hospitalization rate for COVID-19-positive LT recipients is 50%–70%[290]. Immunocompromised individuals above the age of 60 are more prone to develop SARS-CoV-2 infection with protracted viral clearance[291,292]. LT recipients however, are not at increased risk of severity/death as compared to non-LT recipients[44,290]. LT can involve the donor-to-recipient transmission of SARS-CoV-2[293]. COVID-19 is linked to worse postoperative transplant outcomes, particularly in elderly and obese patients[294]. Most LT patients with COVID-19[295,209] should continue to receive systemic immunosuppression with stable dosages, except for immunomodulators[209], as mycophenolate

treatment is considered an independent risk factor for severe COVID-19[289]. The case-fatality rates (17%-18%) are consistent with the expected mortality rates[296]. Those with underlying cancer may require particular consideration[297]. The mortality risk does not change between early (1year) and remote transplantation[296,297].The rate of graft dysfunction during COVID-19 infection is estimated 2.3%-5%[294,298]. The inflammatory reaction in COVID-19 solid organ transplant (SOT) patients is not more severe while IL-6 Levels and incidence of ARDS are lower in SOT suggesting that immunosuppressive medication might limit the COVID-19 hyperinflammation[92].

Hepatocellular carcinoma

Data on HCC patients with COVID-19 infection are limited. COVID-19 in cancer patients is associated with poor outcomes especially if antitumor treatment was received within 14 d[299]. The all-cause mortality in the HCC subgroup is reported 52.4%, almost 7-fold higher than in patients without HCC [238]. COVID-19 patients with HCC may exhibit exacerbated progression with aggravation of existing liver disease[300].

SARS-COV-2 VACCINATION IN CLD

Because neither the adenoviral vector nor the mRNA vaccines contain live or attenuated virus, it is unlikely that vaccination poses a special safety risk for CLD patients. Vaccine trials of both Pfizer-BioNTech[301] and Moderna[302] demonstrated consistent efficacy among subgroups with coexisting conditions, but small numbers of CLD patients were included. It is unknown if SARS-CoV-2 vaccinations are as effective in cirrhotic/transplanted/immunocompromised CLD patients as they are in the general population, especially against quickly evolving viral variations. Preliminary findings suggest that transplant patients are safe[303]. Whilst historically there have been anxieties that vaccination in SOT recipients might develop alloimmunity and graft rejection, no clinical evidence support this concern[44]. LT recipients should be prioritized for immunization since the advantages exceed the risks [44].

LONG COVID-19 LIVER INJURY

Patients who recovered from COVID-19 and followed for months post-infection show increased risk of LFTs abnormalities, suggesting some possible long-term sequelae for the liver[304].The likelihood of persisting liver inflammation and fat deposition (magnetic resonance imaging) following COVID-19 [305] is discussed, as is the prospect of growing liver stiffness over time[306]. In a cohort, ninety randomly selected participants were enrolled three to nine months post-COVID-19 infection and were compared to healthy individuals (negative anti-SARS-CoV-2 immunoglobulin M/immunoglobulin G). The patterns of LI were defined using multiparametric ultrasound (mpUS)[307]. MpUS examination of post-COVID-19 hepatic parenchyma demonstrated higher liver stiffness and steatosis (attenuation imaging-ATI) scores suggestive of LI compared to healthy controls. The most notable change is increased liver stiffness, as measured by greater shear-wave elastography values[307]. Metabolomic analysis of individuals three months after COVID-19 infection reveals higher taurine concentrations, which may be suggestive of LI[308]. Additionally, persistent viral protein and RNA infection of enterocytes[309] in intestinal biopsies for several months after infection renders the small intestine a reservoir of long-term viral replication[34]. Despite mechanisms of long-term LI remain speculative, the sustained endotheliopathy following COVID-19[310] suggests endothelial-mediated inflammation as a possible mechanism. A new entity, post-COVID-19-cholangiopathy, resembling secondary sclerosing cholangitis has also been described in critical COVID-19 cases and typically presents several weeks (mean 118 d) after COVID-19 diagnosis, implying direct LI from COVID-19[311-313].

IMAGING FINDINGS

Increased liver stiffness correlates well with increased ALT/GGT values indicating underlying hepatocellular/cholangiocellular damage on a biochemical level[308]. Patients with increased liver echogenicity and increased ATI values have increased risk for severity (8-fold, 5-fold, respectively). Increased BMI and CRP levels are also associated with hepatic steatosis (ORs = 1.459, 1.387, respectively), while patients with higher steatosis scores present more severe disease[308]. Sonographic findings of ALI, including signs of acute hepatitis and vascular complications, appear in 37.3% of COVID-19 and in 48.7% of ICU COVID-19 patients[314]. CT scan findings are liver hypodensity (26%) and pericholecystic fat stranding (21%).

Table 4 Most frequent liver-related histopathological findings in COVID-19 patients

Findings	Frequency (%)
Portal and sinusoidal microthrombosis	29.4–100
Hepatic/macrovesicular steatosis	50–75
Mild portal inflammation	13.2–66
Centrilobular necrosis	50
Mild acute hepatitis	50
Congestion/dilation of hepatic sinuses	34.7
Portal fibrosis	20.5
Kupffer cell hyperplasia	13.5
Lobular inflammation	11.6
Inflamed cells within the sinusoids (neutrophils, plasmatocytes and Kupffer)	N/A
Panacinar hepatitis, zone 3 necrosis	N/A

N/A: Not applicable; COVID-19: Coronavirus disease 2019.

HISTOPATHOLOGICAL FINDINGS

Most frequently observed liver-related histopathological findings (Table 4) are associated with underlying comorbidities (*e.g.*, NAFLD), rather than of SARS-CoV-2 itself. In initial pathology reports some authors cautioned about the observed "spiked virions", "corona-like" inclusions that could be artifacts of tissue autolysis or of an alternative origin (*e.g.*, intrahepatic cholesterol crystals, lamellations, "crown-like" structures seen in NAFLD, multi-vesicular bodies, exosomes)[12,315,316]. Further investigations found evidence of apoptosis, an abundance of mitosis, mixed inflammatory infiltration in the portal region, and extensive bile duct damage. The hypothesis of direct cell damage was strengthened by identifiable viral particles, viral RNA in the liver, and hepatocytes, together with the intrahepatic detection of SARS-CoV-2 by EM and in-situ hybridization[55-57]. Hepatocytes' cytoplasm contained characteristic coronavirus particles with their spikes, according to ultrastructural analysis[317]. While EM reveals mitochondrial enlargement and apoptosis, in situ hybridization may identify SARS-CoV-2 in 68% of samples[36]. Mitochondrial swelling, endoplasmic reticulum dilatation, and cell membrane dysfunction, document SARS-CoV-2 ability to replicate within hepatocytes[102,317]. Proteomic analysis of autopsy tissue showed mitochondrial dysfunction with dysregulated fatty acid oxidation and oxidative phosphorylation[68]. Biopsies from LT recipients who tested positive for COVID-19 revealed endotheliitis, bile duct damage, and mixed inflammatory portal infiltrates, which are findings of T-cell-mediated rejection[318]. Steatosis is predominant in obesity and overweight patients[102], and its high prevalence is attributed to population's baseline characteristics (severe COVID-19 and steatosis share common risk factors). DILI and CSS may also contribute to the development of hepatic steatosis. Direct vascular damage, portal endotheliitis, portal vein herniation, terminal vessel dilations, and thrombosis with luminal ectasia are examples of acute vascular alterations. Chronic alterations in the portal and sinusoidal vessels, characterized by fibrous thickening of the vascular wall (thrombotic bodies), are sinusoidal inclusions positive for the platelet marker CD61[56,102,108,319]. Lobular ductal pathology is common showing the presence of moderate nuclear pleomorphism in cholangiocytes and canalicular cholestasis[108].

CONCLUSION

This review sheds light on issues raised by early COVID-19 studies concerning discrepancies in prevalence of LI and CLD, and the role of direct SARS-CoV-2 hepatocyte/cholangiocyte injury. The weighty implication of COVID-19-induced LI mechanisms comprising CSS, endotheliopathy/immuno-thromboinflammation, liver hypoxia, and oxidative stress with respect to histopathological and immunohistochemical findings is meticulously discussed. Finally, an emerging entity, long-COVID-19 persistent LI is also studied.

FOOTNOTES

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Ferroptosis regulates key signaling pathways in gastrointestinal tumors: Underlying mechanisms and therapeutic strategies

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Abstract

Ferroptosis is an emerging novel form of non-apoptotic, regulated cell death that is heavily dependent on iron and characterized by rupture in plasma membrane. Ferroptosis is distinct from other regulated cell death modalities at the biochemical, morphological, and molecular levels. The ferroptotic signature includes high membrane density, cytoplasmic swelling, condensed mitochondrial membrane, and outer mitochondrial rupture with associated features of accumulation of reactive oxygen species and lipid peroxidation. The selenoenzyme glutathione peroxidase 4, a key regulator of ferroptosis, greatly reduces the lipid overload and protects the cell membrane against oxidative damage. Ferroptosis exerts a momentous role in regulating cancer signaling pathways and serves as a therapeutic target in cancers. Dysregulated ferroptosis orchestrates gastrointestinal (GI) cancer signaling pathways leading to GI tumors such as colonic cancer, pancreatic cancer, and hepatocellular carcinoma. Crosstalk exists between ferroptosis and other cell death modalities. While apoptosis and autophagy play a detrimental role in tumor progression, depending upon the factors associated with tumor microenvironment, ferroptosis plays a decisive role in either promoting tumor growth or suppressing it. Several transcription factors, such as *TP53*, activating transcription factors 3 and 4, are involved in influencing ferroptosis. Importantly, several molecular mediators of ferroptosis, such as p53, nuclear factor erythroid 2–related factor 2/heme oxygenase-1, hypoxia inducible factor 1, and sirtuins, coordinate with ferroptosis in GI cancers. In this review, we elaborated on key molecular mechanisms of ferroptosis and the signaling pathways that connect ferroptosis to GI tumors.

Key Words: Ferroptosis; Gastrointestinal cancers; Nuclear factor erythroid 2–related factor 2; Apoptosis; Autophagy

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Core Tip: Gastrointestinal tumors contribute to the majority of cancer-related deaths. Ferroptosis is a novel form of non-apoptotic cell death that plays a vital role in reducing the invasiveness and metastasis of gastrointestinal tumors. Herein, we discussed the regulatory mechanisms involved in ferroptosis through the hallmark pathways of glutathione peroxidase 4, iron metabolism, lipid peroxidation, and redox signaling that provide a novel therapeutic approach for gastrointestinal cancers.

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INTRODUCTION

Cancer is a notorious disease that causes numerous disorders and deaths worldwide, particularly gastrointestinal (GI) cancers comprising esophageal cancer, gastric cancer (GC), liver cancer, pancreatic cancer, and colorectal cancer[1]. A recent report stated that there are approximately 4 million new cases each year, which is far greater than breast and lung cancers combined; thus, there is a need to improve the therapeutics of GI cancers[2]. Although there has been an improvement in the prognosis of GI cancers in the last decade due to development of intensive therapies, such as incorporating cytotoxic drugs and targeted therapies, GI cancers are one of the leading causes of cancer deaths in developed and developing countries[3-5]. With varying risk factors, incidence, prevalence and prognosis, early diagnosis with increased screening may facilitate therapies to fight against GI cancers.

Biomarkers, including epigenetic markers, also play an effective role in decreasing the progression of GI cancers by early diagnosis and decreasing risk assessment by predicting the tumor response from specific therapies in patients[6]. Apart from internal stimulation, external exposures, such as dietary intake, tobacco use, alcohol consumption, obesity, and pathogens, are considered to increase the risk of GI tumors[7]. Various improvements in the therapeutic aspects to decrease the burden of GI cancer, including the chemoprevention of these cancers by using antioxidants, have drawn much attention[8]. Murphy *et al*[9] estimated that by 2025, the pervasiveness of obesity will be increased in men and women by 18% and 21%, respectively, which could escalate the encumbrance of GI cancers worldwide [9].

The induction of cell death by ferroptosis has increased in the recent past. Cell death is an endogenous mechanism that regulates homeostasis, and it is perceived as a requisite bodily process. As an imperative system, it exterminates useless or unwanted cells and bolsters the defense system of the body. Many forms of cell death have been observed in the recent past, particularly accidental cell death and regulated cell death (RCD)[10,11]. Accidental cell death occurs suddenly in response to stress, heat shock, or mechanical nature, while RCD is orchestrated by exhibiting a precise signaling cascade provided by a defined group of effector molecules. Many forms of RCD have been observed, such as apoptotic, autophagic, pyroptotic, necrotic, entotic, and ferroptotic cell death[12].

Investigating RCD introduces a new path for developing cancer therapeutics. Among the various forms of RCD, ferroptosis, an iron dependent, non-apoptotic RCD, is fascinating because of its ability to manage cancer cells that develop resistance to apoptosis and drugs. Although a link between iron and lipid peroxidation has been found in cancer research, this form of RCD has a peculiar combination of morphological, biochemical, and genetic characteristics distinct from necrosis, apoptosis, and autophagy [13]. Dixon *et al*[14] proposed the term ferroptosis in 2012, and its emerging role in several other disease settings was observed[14]. Ferroptotic cell death is characterized by the occurrence of oxidative stress and membrane lipid peroxidation, thereby causing mitochondrial atrophy, increased density of the mitochondrial membrane, and membrane damage[15]. Therefore, this review summarizes the role of ferroptosis in regulating key cancer signaling and cell death pathways in GI tumors for better prognosis.

GASTROINTESTINAL CANCERS: INCIDENCE AND MORTALITY

GI cancers contribute to significant morbidity and mortality rates worldwide[16] (Table 1), accounting for 26% of all cancer-related incidence and 35% of all cancer-related fatalities. It was noted that there is a two-fold increase in men compared to women. It has been reported that by 2040, there will be an increase in new cases and deaths from GI cancers by 58% and 73% to 7.5 million and 5.6 million, respectively[17]. Incidence and mortality rates of GI cancers worldwide is presented in Table 1[18-22].

Table 1 Incidence and mortality rates of gastrointestinal cancers worldwide

No	Gastrointestinal cancers	Incidence	Mortality	Ref.
1	Esophageal cancer	604100	544100	[18]
2	Gastric cancer	1100000	770000	[19]
3	Liver cancer	905700	830200	[20]
4	Pancreatic cancer	495773	466003	[21]
5	Colorectal cancer	1930000	900000	[22]

While cell death modalities play a decisive role in eradication of tumor cells and maintenance of homeostasis, the role of RCD pathways, particularly ferroptosis, in tumor progression and metastasis of cancers has been the subject of interest for the last decade.

DISTINCTIVE FEATURES OF FERROPTOSIS

The various features of ferroptosis include the morphological, biochemical, and epigenetic alterations that describe this novel form of cell death.

Morphological distortion of ferroptosis

Ferroptosis is a non-apoptotic form of cell death because it lacks the classical features of apoptosis, such as mitochondrial cytochrome-C release, caspase activation, and chromatin fragmentation[23]. In contrast, ferroptosis induces mitochondrial membrane disintegration, which results in cell enlargement, plasma membrane rupture, volume reduction, increased density, and disappearance of cristae observed under the electron microscope. These changes are potentially caused by permeability loss due to increased lipid peroxidation[24].

Biochemical changes involved in ferroptosis

The main biochemical features of ferroptosis are iron accumulation, reactive oxygen species (ROS) production, and induction of lipid peroxidation. Iron is the most abundant metal and is essential to all life on earth. As an iron-dependent form of cell death, increased circulating iron leads to the Fenton reaction through activated iron-containing enzymes that act as biochemical markers of ferroptosis[25]. Intracellular iron and iron-containing enzymes are indispensable for the execution of ferroptosis. Molecular regulators associated with iron homeostasis, such as transferrin, lactotransferrin, transferrin receptor, nuclear receptor coactivator 4-dependent degradation of ferritin, iron regulatory protein, and iron responsive element regulatory network, facilitate ferroptotic cell death[26,27].

ROS are the major culprits in many disease settings. ROS can damage essential biomolecules such as DNA, protein, carbohydrates, and lipids, thereby causing denaturation, peptide s-s bond breaking, crosslinking, enzyme inhibition, and permeability changes in tissues and cells. Therefore, increased ROS promote several perturbances leading to altered cell death pathways as well as induction of ferroptosis [28].

Lipid peroxidation is a seminal process in which the generated free radicals (H_2O_2, super peroxides, peroxy radicals) target long-chain fatty acids[29]. Extensive lipid peroxidation affects the permeability, fluidity, and curvature of the membrane, thereby stimulating cell death by forming micelles and pores in the biological membrane. A close connection between lipid peroxidation and ferroptosis exists in cancers. Importantly, to overcome drug resistance in chemotherapy, ferroptosis plays a crucial role in tumor microenvironment, thereby controlling cell proliferation through redox signaling pathways[30].

Epigenetic alterations

Epigenetics is a genetic mechanism that can influence gene expression by DNA methylation, histone modifications, and the effects of noncoding RNA without altering the DNA sequence[31,32]. DNA methylation is an epigenetic modification process that uses DNA methyltransferases to covalently transfer the methyl group to the C-5 position in the cytosine ring of DNA[33]. It has been reported that hypermethylation of the *CDH1* gene promoter could increase ferroptosis susceptibility in head and neck cancer cells[34]. Recently, epigenetic regulation through H2B monoubiquitination and p53 has been determined[35].

Histone acetylation is controlled by histone acetyltransferases, bromodomains (BRDs), and histone deacetylases (HDACs) to regulate ferroptosis. In the histone acetylation process, histone acetyltransferases play the writer role, BRDs the reader role, and HDACs the eraser role[36,37]. The tumor suppressive role of *Tp53* is well known. For p53-induced ferroptosis, acetylation plays a crucial role by regulating solute carrier family 7 member 11 *(SLC7A11)* expression[38]. Acetylated and mutant $p53^{3KR}$

suppresses *SLC7A11* expression and inhibits cysteine uptake, which alleviates ferroptosis and lipid peroxidation by decreasing glutathione (GSH) synthesis[38,39]. BRD4 induces the expression of anti-ferroptotic genes, and it has been observed that the BRD4 inhibitor JQ1 induces ferroptosis by downregulating the expression of ferroptosis-related genes such as glutathione peroxidase 4 (*GPX4*), *SLC7A11*, and solute carrier family 3 member 2 in breast and lung cancer cells[40]. HDACs were initially identified as an eraser for removing acetyl groups from histones, but it was later discovered to be involved in many important biological functions. Recently, HDAC inhibitors have been endorsed as potential therapeutics for various cancers[41].

As a multifarious group of noncoding transcripts, noncoding RNAs, microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs were first considered nonfunctional junk, but they participate in a broad domain of gene-regulatory pathways[42]. Beyond this, it has been suggested that circular RNAs play a role in ferroptosis by regulating multiple signaling pathways either directly or indirectly by acting on the key regulating factors and upstream targets of ferroptosis[43]. miRNAs tend to act as stimulants in ferroptosis by targeting ferroptosis-associated factors by downregulating the expression of activating transcription factor 4 (ATF4), which is a stress signal that tends to inhibit ferroptosis by miR-214-3p in hepatocellular carcinoma (HCC) by inducing *SLC7A11* and several other miRNAs, including miR-101-3p, at high expression levels. They profoundly enhance the activity of nuclear factor kappa B, which regulates ferroptosis *via* GPX4 and prostaglandin endoperoxide synthase 2 in lung cancer, and miR-324-3p at high expression levels induces ferroptosis in lung adenocarcinoma by targeting GPX4[44]. Furthermore, miRNAs have also been found to have inhibitory effects on ferroptosis; therefore, they play diverse roles in ferroptosis. The alterations they cause to ferroptosis-associated factors make them a potential target in cancer therapeutics.

FERROPTOSIS INDUCERS

Over the past few years, several small molecules and plant-based compounds that target transporters or enzymes involved in ferroptosis have been described.

In principle, GSH synthesis and GPX4, a selenoenzyme, are the major regulators of ferroptosis. GSH is an important substrate for GPX4. Therefore, any depletion in GSH leads to substantial lipid peroxidation, leading to ferroptosis. By inhibiting GPX4, accumulation of lipid peroxidation takes place [45]. To produce GSH, system Xc^- regulated cysteine activity is needed. Certain compounds, such as sorafenib, glutamate, and erastin, induce ferroptosis through inhibition of system Xc^-. Ras-selective lethal 3 (RSL3), a known GPX4 inhibitor and other compounds containing electrophilic chloroacetamides, covalently binds and restricts selenocysteine activity inside the active site of GPX4 to initiate ferroptosis[46]. Other nitrile oxide electrophiles include ML210, JKE-1674, and JKE-1716, which attach to selenocysteine and cause ferroptosis[46,47]. By directly oxidizing lipids and indirectly impairing GPX4 action, FINO2 causes ferroptosis, and FIN56 drives the destruction of GPX4 to induce ferroptosis[48]. To induce ferroptosis, FINO2 harnesses either a direct or indirect iron oxide to induce suppression of GPX4 activity. Organic peroxides are compounds with multiple O_2 bonds that are cleaved, resulting in the production of RO anions. These organic peroxides are often used as models to induce ROS. A commonly held view is that the lipid peroxide analogue tert-butyl hydroperoxide stimulates lipid peroxidation-dependent ferroptosis[49].

Excessive iron accumulation is a precursor of ferroptosis in a plethora of cell types. *In vitro*, hemoglobin causes ferroptosis, and *in vivo*, it causes intracerebral hemorrhage in other disease states[50-52]. Furthermore, the rise in cellular iron levels and consequent ferroptosis caused by pharmacological stimulation of selective autophagy through degradation of ferritin is known as ferritinophagy[53,54]. Apart from these, there are many other ferroptotic inducers; for example, in human pancreatic cancer cells, zalcitabine causes autophagy-dependent ferroptosis, pointing to a link between DNA sensor pathways, autophagy activation, and mitochondrial malfunction[55].

FERROPTOSIS INHIBITORS

The inhibition of ferroptosis by small molecular compounds takes place through the following mediators.

Iron chelators: The important role of iron is to promote lipid peroxidation by either activating iron-containing lipid oxygenases (LOX) or nonenzymatic iron-mediated Fenton action[56-58]. Iron chelators like deferoxamine inhibit ferroptotic cell death. Iron chelators reduce H_2O_2-induced necrosis but not ferrostatin-1, suggesting that iron may be potentially involved in multiple cell death modalities[59,60].

Enzyme inhibitors: The enzyme inhibitor acyl-CoA synthetase long chain family member 4 (ACSL4) mediates addition of CoA to long-chain fatty acids, particularly arachidonic acid, and appears to be a crucial precursor to further arachidonate lipoxygenase-dependent lipid peroxidation[57]. ACSL4 and

arachidonate lipoxygenase inhibitors are known to inhibit the ferroptotic cell death process by preventing LOX accumulation[57,61]. In addition, NAD phosphate oxidase inhibitors comprising of diphenylene iodonium and GKT137831 prevent erastin-induced ferroptosis[62].

Protein degradation inhibitors: GPX4 may be degraded by a variety of ferroptosis activators, which results in lipid peroxidation. However, FIN56 and erastin-induced GPX4 breakdown is inhibited by the molecular chaperone heat shock protein 90, 5-(tetradecyloxy)-2-furoic acid, and dopamine[63-65].

Other inhibitors: The mitogen-activated protein kinase kinase inhibitor, U0162 is commonly used to reduce ferroptosis owing to its broad antioxidant activity[66]. The strong inhibitory effects of exogenous monounsaturated fatty acids (MUFAs) or deuterated polyunsaturated fatty acids (PUFAs) on ferroptosis may be attributed to their displacement of PUFAs from phospholipids, which lowers the accumulation of lipid peroxidation[67].

REGULATION OF FERROPTOSIS

A deeper knowledge of how ferroptosis is regulated by metabolic pathways, including iron, GSH, and lipid metabolism, has been the focus of research in proposing therapeutic drugs. In this context, several metabolic and cancer signaling pathways that connect ferroptosis have been described.

GPX4

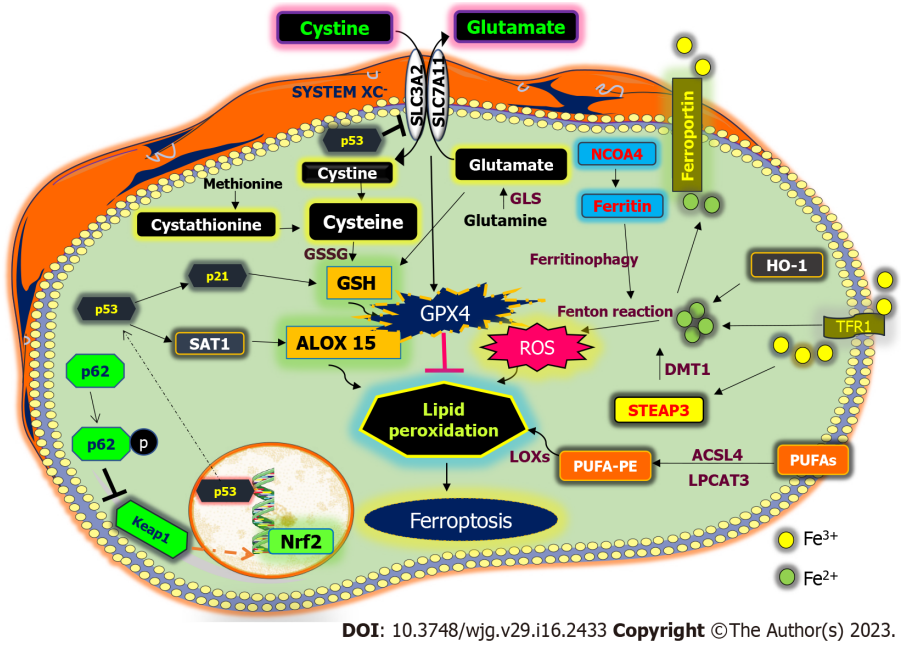
Ferroptosis is primarily caused by the inactivation of the cellular antioxidant system, particularly the system that is dependent on the antioxidant defense Xc^- GSH-GPX4 that leads to toxic lipid ROS accumulation[68]. GSH peroxidases are pivotal enzymes that enable the reduction of peroxides *via* GSH. The system Xc^- family mediates transport of cysteine and glutamate, where glutamate is exported outside the cell and cysteine is imported inside the cell, which initiates the production of GSH, hence inhibiting ferroptosis in cancer cells[69] (Figure 1). The GPX protein comprises different types, among which GPX4, a selenoprotein, is an elemental antioxidant mediator known for its capability of reducing large peroxides such as PUFAs[68]. It is primarily involved in the maintenance of lipid metabolism and defense against the accumulation of toxic lipid ROS. Since GPX4 activity is directly impacted by conditions that reduce intracellular cysteine and subsequently GSH levels and because GSH is the dominant antioxidant in mammalian cells, these conditions trigger ferroptosis[70]. Small molecules such as erastin can block GSH or GPX4 expression to activate ferroptosis. In many cells, the rate at which GSH is synthesized is limited by the internal reduction of cystine to cysteine[71].

Lipid metabolism

The process in which free radicals and non-free radicals cleave the C-C double bonds in lipids such as PUFAs, phospholipids, glycolipids, and cholesterol is known as lipid peroxidation.

According to lipidomic research, arachidonic acid and adrenic acid are key driving factors in inducing ferroptosis. Elongation of very long-chain fatty acid protein 5 and fatty acid desaturase 1 are associated with the synthesis of fatty acids, where both enzymes are upregulated in mesenchymal GC cells, resulting in ferroptosis sensitivity, according to studies on the disease[72]. The process by which these PUFAs become coenzyme-A derivatives that are integrated into phospholipids triggers ferroptosis. Both autophagy and deubiquitylation can result in the reduction of intracellular GPX4[73]. Specialized sequences in GPX4 are distinctly recognized by heat shock protein 90 and facilitate the breakdown of GPX4 by chaperone-mediated autophagy[63]. On the other hand, the inhibition of chaperone-mediated autophagy by the mTOR pathway is relieved by its suppression, which may have caused GPX4 to degrade and cause ferroptosis[74].

Numerous investigations have revealed ACSL4 as a crucial sensitizer in ferroptosis machinery. ACSL4 mediates the esterification of PUFAs into phospholipids by adding CoA to the polyunsaturated bond of arachidonic acid[75] (Figure 1). Lysophosphatidylcholine acyltransferase 3, which is activated by ACSL4, contributes to ferroptotic lipid signaling by adding acyl groups to lysophospholipids, particularly to the phospholipids phosphatidylcholine and phosphatidylethanolamine[75]. Apart from PUFAs, MUFAs can promote ferroptosis resistance by initiating mutations in the PI3K-AKT-mTOR pathways, sterol regulatory element-binding protein 1 can mediate the production of MUFAs, which aids cancer cells in resisting ferroptosis[76]. MUFAs can modulate the hydroxycarboxylic acid receptor 1-sterol regulatory element-binding protein 1-stearyl-coenzyme A desaturase 1 pathway and lactate in the tumor microenvironment, which might increase the production of MUFAs and lower the expression of ACSL4 to avoid ferroptosis[77]. As functional lipids in ferroptosis, polyunsaturated ether phospholipids have a significant regulatory effect in changing cancer cells from a ferroptosis-sensitive state to a ferroptosis-resistant state[78]. Thus, lipid metabolism has a notable effect on the capability of cancer cells to induce ferroptosis.



DOI: 10.3748/wjg.v29.i16.2433 **Copyright** ©The Author(s) 2023.

Figure 1 Mechanistic insights of ferroptosis regulated pathways are represented. Glutathione peroxidase 4 (GPX4) is central in ferroptosis and works through the initiation of system Xc^-, which mediates the export of glutamate and import of cystine. Cystine is required for the synthesis of glutathione (GSH). p53 induces the expression of p21 following oxidative stress and further induces GSH leading to GPX4 expression. Alternatively, p53 increases the expression of spermidine/spermine N1-acetyltransferase 1 (SAT1), which serves as the transcriptional target of p53. SAT1 joins ferroptotic pathway through induction of arachidonate lipoxygenase (ALOX) 15 leading to lipid peroxidation. In addition, p62 phosphorylation targets Kelch-like ECH-associated protein 1 (Keap1) leading to accumulation of nuclear factor erythroid 2–related factor 2 (Nrf2) in the nucleus to subscribe antioxidant enzymes during oxidative stress. Acyl-CoA synthetase long chain family member 4 (ACSL4) is an isozyme catalyzes polyunsaturated fatty acids (PUFAs) contributes to ferroptosis through induction of lipoxygenases. Ferritinophagy induction *via* ferritin contributes to the Fenton reaction leading to accumulation of reactive oxygen species (ROS) and lipid peroxidation. DMT1: Divalent metal transporter 1; GSSG: Glutathione disulfide; HO-1: Heme oxygenase-1; LOX: Lipid oxygenase; LPCAT3: Lysophosphatidylcholine acyltransferase 3; PUFA-PE: Polyunsaturated fatty acid-phosphatidylethanolamine; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; STEAP3: Six-transmembrane epithelial antigen of the prostate 3; TFR1: Transferrin receptor 1.

Iron metabolism

To carry out ferroptosis and allow lipid peroxides to build up, iron is particularly necessary. The capacity of iron to catalyze numerous metabolic events and flip between the various ionic states depends largely on how it can absorb and give electrons[79]. In the Fenton reaction, Fe^{2+} is converted to Fe^{3+} in the presence of H_2O_2, and $HO^{\cdot}$ is produced as a result of electron transfer to H_2O_2[80]. In contrast, the Haber-Weiss process could convert Fe^{3+} back to Fe^{2+} by reacting with O_2, causing O_2 to lose one electron and become O^{2-}[81].

The transfer of iron takes place in the following ways. Transferrin synthesized by the liver as a chelator of Fe^{3+} ions. In contrast to apo-transferrin, which is sans iron transferrin that is not recognized by transferrin receptor 1 (TfR1) and not internalized, transferrin recognizes and binds to TfR1 when it reaches the cell membrane, which is then internalized by clathrin-mediated endocytosis[82]. Accumulating evidence suggests the role of iron metabolism in ferroptosis[83,84]. Fe^{3+} is liberated from the transferrin TfR1 complex due to the acidic pH of endocytic vesicles and is converted to Fe^{2+} by the six-transmembrane epithelial antigen of the prostate (STEAP) family. STEAP1 and STEAP2 are implicated in multiple human malignancies, including Ewing sarcoma bladder, ovary, colon, breast, prostate, pancreatic, and cervical cancer[85-87]. Malignant gliomas have high levels of STEAP3, which triggers the cancer epithelial-mesenchymal transition[88]. Under hypoxic circumstances, STEAP4 is activated, resulting in an imbalance of mitochondrial iron and increased ROS generation[89].

The intracellular labile iron pool is subsequently created by divalent metal transporter 1, which mediates the release of Fe^{2+} into the cytoplasm[90]. Apo-transferrin is released back to the plasma membrane post recycling, while TfR1 and apo-transferrin remain linked[91]. Fe^{2+} is then directly transported into cells by divalent metal transporter 1 during the conversion of Fe^{3+} to Fe^{2+} (Figure 1). Another mechanism includes the absorption of porphyrin-bound Fe^{2+} that contains hemoglobin, particularly in macrophages, and involvement of cell membrane receptors of iron-storing protein ferritin, such as scavenger receptor class A member 5, as observed in kidney and embryonic development, which absorbs iron to regulate the ferroptosis mechanism[92,93].

Role of ferritin in Fe transport

Ferritin, an iron-sequestering protein with 4500 iron atoms, plays a crucial role in iron transport, cellular multiplication, angiogenesis, and immune suppression[94]. By using NCOA4, ferritin can also be split

up to liberate free iron, through "ferritinophagy"[95]. Ferroportin (FPN), recognized as the sole iron release pump that works with ceruloplasmin to export iron, is primarily in charge of moving Fe^{2+} out of cells[96]. Ceruloplasmin controls HepG2 and Hep3B cell iron regulation to prevent ferroptosis, and its loss causes a buildup of intracellular Fe^{2+} and lipid ROS and enhances ferroptotic death caused by erastin and RSL3[97]. Prominin-2 promotes the growth of multivesicular structures that comprise ferritin and exosomes that transfer iron from cells, enabling ferroptosis resistance in breast cancer[98]. FPN is severely reduced in many cancer types, suggesting that cancer cells may contain large amounts of iron[99]. Decreased FPN levels promote proliferation and epithelial-mesenchymal transition in triple-negative breast cancer cells[100]. Hepcidin, produced by tumors of the liver, promotes the breakdown of FPN and aids in the spread and development of cancer[101].

TRANSCRIPTION FACTORS

Activating transcription factor 3 and activating transcription factor 4

The unfolded protein response is activated in mammalian cells due to endoplasmic stress and has a two-dimensional functional role in cell survival and death[102]. Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP-responsive element-binding protein family of transcription factors. It binds to the ATF/cAMP-responsive element-binding protein cis-regulatory element and coordinates gene expression. ATF3 has tumor suppressive roles and inhibits cancer malignancy in GI cancers[103]. The context-dependent role that ATF3 plays in cancer is likely due to complex protein-protein interaction networks in which ATF3 is involved.

Indeed, in addition to transcriptional regulation, ATF3 has been found to interact with many critical cellular proteins and regulate their functions. One of the well-characterized ATF3-binding proteins is wild-type p53, and ROS signals are necessary for this aberrant production of endoplasmic reticulum stress markers; however, the antioxidant N-acetyl L-cysteine prevents overexpression and consequent ferroptotic cellular death. Although the ATF3-TP53 complex helps to initiate DNA damage, *TP53* is not necessary for ATF3-regulated suppression of *SLC7A11* transcription[104]. Nuclear factor erythroid 2-related factor 2 (Nrf2) can, in contrast, regulate the expression of ATF3 by creating complex feedback loops for the activation of a number of transcription factors in coordinating the ferroptotic response [105].

ATF4 is a double-sided sword that plays a dual role in ferroptosis. In numerous cancer cells, including human glioblastoma as well as pancreatic cancer cell lines, the depletion of ATF4 increases erastin-induced or RSL3-induced ferroptosis, and the inhibition of ATF4 increases artesunate-induced ferroptosis in the DAUDI cell line[106,107]. The classes of genes targeted by ATF4, such as *SLC7A11*, heat shock protein 5 (*HSPA5*), and tribbles pseudokinase 3, may have an impact on this ATF4-dependent mediation of ferroptosis in various cancer types. ATF4 is inhibited by the erastin-induced overexpression of HSPA5, which results in the sequential breakdown of GPX4 and lipid peroxidation [108-112]. Thus, ATF3-dependent and/or ATF4-dependent pathway dysregulation might influence ferroptosis in a tumor-specific phenotype.

Nrf2

Binding to antioxidant response elements, Nrf2, a transcription factor from the Cap-N-Collar family, is essential in regulating antioxidant genes and maintaining redox homeostasis[113]. Nrf2 transcriptional activity is normally attached to Kelch-like ECH-associated protein 1 (Keap1), retaining it in the cytoplasm. Nrf2 separates from Keap1 during environmental conditions (such as oxidative stress) and subsequently translocates into the nucleus, where it stimulates the expression of ARE-dependent target genes[113]. Nrf2, however, depends on the expression of the target genes that are involved in the control of cell proliferation, migration, and death to have a double impact on carcinogenesis and tumor treatment[114].

Initially, researchers showed that ferroptosis resistance may be promoted by activating the Nrf2 pathway using a model of HCC. In human HCC cell lines, ferroptosis activators like erastin and sorafenib boost Nrf2 stability by inhibiting the development of the Nrf2-Keap1 complex. The autophagy receptor SQSTM1/p62 elevates Nrf2 expression by inactivating Keap1, which is another regulator of this process[115]. By boosting GSH production and function, Nrf2-dependent genes such as glutathione synthetase, GPX4, and *SLC7A11* contribute to ferroptosis inhibition[115]. Control of NADPH synthesis, a vital electron donor required for reduction of oxidized substrates[116], which is also a ferroptosis sensitivity biomarker[117], is another way that Nrf2 intermediate metabolism is connected to the regulation of ferroptosis. Overall, Nrf2 is an important transcription factor that regulates ferroptosis.

TP53

p53, a tumor suppressor encoded by the *TP53* gene, is involved in the mediation of DNA damage, oncogene activation, and hypoxia. p53 promotes ferroptosis by transcriptional or posttranscriptional pathways in addition to its impacts on apoptosis, autophagy, and cell cycle. p53 can both induce and inhibit ferroptosis in a context dependent manner. To cause ferroptotic cell death, p53 increases the

production of spermidine/spermine N1-acetyltransferase 1, which sequentially causes increased 15-LOX expression responsible for oxidation of PUFAs (Figure 1)[118]. Simultaneously, p53 decreases the expression of ELAV-like RNA-binding protein and its action with LINC00336, by limiting the capability of cells to fight ferroptosis by increasing the activity of cystathionine β synthase (CBS)[119]. Additionally, p53 interaction with ubiquitin-specific protease 7, a deubiquitinating enzyme, facilitates its nuclear translocation by altering histones, favorably controlling ferroptosis[120]. Human colon cancer cells, such as HCT116 and SW48, are inhibited against ferroptosis by p53 in a transcription-independent manner[121] The p53-p21 axis prevents ferroptosis, allowing cancer cells to withstand stressful metabolic situations. Thus, this dual role of p53 in ferroptosis can be explored for therapeutic treatment of cancers.

Heme oxygenase-1

Heme oxygenase-1 (HO-1) is a significant redox-mediating enzyme that is activated in reaction to oxidative stress, cellular stress, neurodegeneration, and other diseases. HO-1 has a dual personality. The use of HO-1 antagonist zinc protoporphyrin IX and HO-1 knockdown animals demonstrated that HO-1 increases erastin-induced ferroptosis[121]. Conversely, because HO-1 expression is knocked down, erastin and sorafenib more effectively limit cell proliferation in HCC caused by these drugs[121]. In addition, HO-1 mediates iron and ROS levels where Nrf2-derived HO-1 provides a cytoprotective effect by scavenging ROS when HO-1 is moderately active[121]. In contrast, given that cancer cells produce more HO-1 than normal cells, a high level of HO-1 activation may increase fragile Fe^{2+}, resulting in an excess of ROS and eventual oxidative cell death[121]. Hence, the employment of ferroptosis by HO-1 activation can define the fate of cancer cells.

Sirtuins

Sirtuins are NAD^+-dependent deacetylases that are involved in DNA repair, cellular metabolism, and cancer development. As a key oxidizing agent, sirtuins tend to play a crucial role in regulating redox signaling pathways[122]. Seven sirtuins have been identified in mammals so far, and each family has been found to regulate cellular homeostasis. SIRT1 activation increases the antioxidant activity mediated by oxidative stress-related transcription factors[122]. Studies have found that increased expression of SIRT1 induces Nrf2-mediated antioxidant activity, thereby increasing GPX4 and GSH levels in HCC. Conversely, their downregulation by protocadherin 20 mediates ferroptotic cell death by lowering the expression of GPX4 and GSH and increasing intracellular iron levels and ROS[123]. SIRT6 promotes ferroptosis in pancreatic cells through upregulation of the ROS levels[124], and its downregulation causes GC cells to resist sorafenib-induced ferroptosis[125].

Hypoxia inducible factor

Hypoxia is a prominent factor involved in the progression and metastasis of numerous cancers. Prolyl hydroxylase hydroxylates the hypoxia inducible factor (HIF)-a subunits HIF-1α and HIF-2α under normoxic circumstances; subsequently, they are subjected to ubiquitin-mediated proteolysis and destruction. HIF target genes are activated in hypoxia as a result of HIF-1α and HIF-2α failing to hydroxylate and translocate into the nucleus[126]. Our laboratory's previous studies found that under hypoxic conditions, hypoxia-inducible factors such as HIF-1α and HIF-2α are activated and stimulate the activation of matrix metalloproteinases 2 and 9, thereby increasing tumorigenesis[127]. Singhal *et al*[128] demonstrated the mechanism by which activation of HIF-2α raises cellular iron to accelerate ferroptosis or irreversible cysteine oxidation that causes cell death.

FERROPTOSIS AS A NOVEL TARGET FOR GI CANCER RESEARCH

The leading cause of cancer deaths and incidence from GI tumors has become the most essential health concern, so improving the therapeutic aspects provides an important way to decrease the burden of GI cancers. Several studies have used ferroptotic cell death as a key modulator to inhibit the growth of GI cancers such as colorectal cancer, liver cancer, pancreatic cancer, GC, and esophageal cancer.

Ferroptosis in colorectal cancer

Recent studies by Wang *et al*[129] suggested that ferroptosis can have a potential modulatory role in the targeted killing of chemoresistant colon cancer cells, and ferroptosis-related genes could also be harnessed as biomarkers in colorectal cancer therapeutics. Genes involved in the colorectal cancer tumor microenvironment are intricately linked to ferroptosis, and some of them are involved in the lipid peroxidase and GPX/GSH enzyme system. Iron metabolism genes are also powerful prognostic markers in colorectal cancer; for example, elevated expression of thio-redoxin tumor suppressing protein is closely concomitant with iron accumulation in mitochondria[130]. The above findings clearly state that targeting ferroptosis-inducing genes could provide a novel avenue for treating colorectal cancer. p53 tumor-suppressing protein and heme oxygenase have been implicated as key factors in regulating ferroptosis in colorectal cancer.

Ferroptosis in liver cancer

HCC is the most prevalent type of liver cancer, accounting for approximately 90% of cases[131]. Multiple studies have reported that ferroptosis plays a role in ameliorating the burden of HCC progression. Sorafenib, an anticancer agent that is used for the treatment of advanced HCC, has been found to induce ferroptosis by initiating the translation of rapamycin kinase signaling pathway, thereby initiating ferroptosis. Iron chelators such as deferoxamine inhibit sorafenib-induced ferroptosis by reducing the oxidative stress created by sorafenib in HCC cells[132].

The finding of a potent inhibitor of CBS, which is the main source of cystathionine gamma lyase by metabolizing homocysteine to cystathionine to increase intracellular L-cysteine, called CH004, selectively inhibits CBS but not human cystathionine gamma lyase in both *in vivo* and *in vitro* experiments, thereby increasing lipid ROS and decreasing the viability of HepG2 cells, indicating their role in ferroptosis[133].

Modulating the function of key regulators of ferroptosis also plays a significant role in the induction of ferroptotic cell death in HCC cells. Nrf2 is a key regulator of the antioxidant response. It plays a negative regulatory role in ferroptosis by actuating p62-Keap1-Nrf2 pathway, which upregulates the expression of quinone oxidoreductase 1, HO-1, and ferritin heavy chain 1. Therefore, inhibiting the p62-Keap1-Nrf2 pathways by erastin and sorafenib significantly enhances the ferroptosis-mediated cell death of liver cancer cells[134].

The mitochondrial membrane protein CDGSH iron sulfur domain 1 serves as a target to treat diabetes using glitazone, but it acts as a negative regulator of ferroptosis by modulating mitochondrial iron uptake; therefore, the pharmacological or genetic inhibition of CDGSH iron sulfur domain 1 enhanced mitochondrial lipid peroxidation, increasing erastin-induced ferroptosis in liver cancer cells[135].

The highly reactive metabolite NAPQ1 from acetaminophen, an analgesic and antipyretic agent, is involved in the ferroptotic cell death of HepG2 cells by decreasing their viability through GSH depletion and GPX inhibition[136]. The expression of retinoblastoma protein (Rb) in HCC cells determines the susceptibility of cancer cells to sorafenib treatment and regulates ferroptosis. Louandre *et al*[137] showed that the decrease in the levels of Rb protein exhibits an increase in cell death when cells were treated with sorafenib compared with controls; thus, determining the Rb status of HCC patients treated with sorafenib will provide novel insight into the HCC treatment.

LncRNAs represent a vital class of molecules that have regulatory effects in both physiological and abnormal conditions, but lncRNA GABPB1-AS1 has a role in regulating oxidative stress, confirming its involvement in the ferroptosis-mediated cell death of HCC cells. The expression of this lncRNA was upregulated by erastin to inhibit the translation of GA binding protein transcription factor subunit beta 1, resulting in the rapid accumulation of ROS in HepG2 cells by decreasing the expression of the *PRDX5* peroxidase gene[138].

Ferroptosis in pancreatic cancer

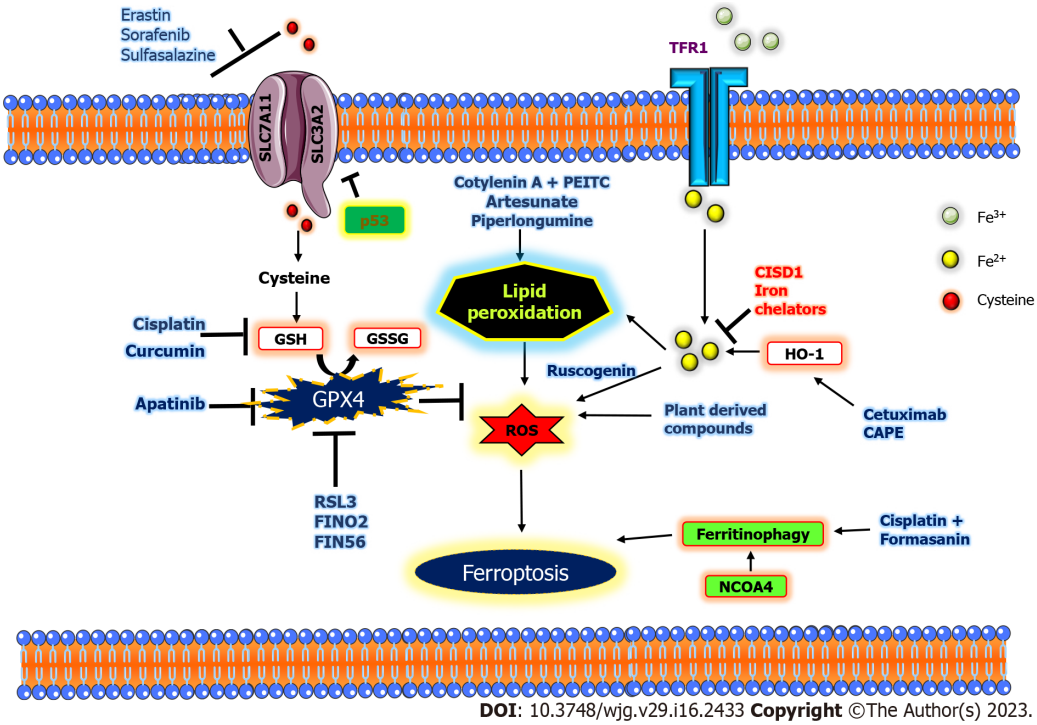
Several molecules were demonstrated to induce ferroptosis in pancreatic cancer cells in which the first-line drug gemcitabine is used to treat advanced pancreatic cancer, but HSPA5 causes resistance to gemcitabine treatment by inhibiting ferroptosis. Therefore, genetically or pharmacologically inhibiting HSPA5 enhanced the sensitivity of gemcitabine to pancreatic ductal adenocarcinoma cells by inducing ferroptosis[139]. Inhibiting cytosolic aspartate aminotransferase, which is predominant for redox balance, represses the growth of pancreatic cancer cells by enhancing labile iron release, thereby inducing sensitivity to ferroptosis[140].

Several studies have shown that certain natural plant extracts possess potential anticancer effects by inducing ferroptosis in pancreatic cancer cells. A saponin called ruscogenin represses cell viability and induces ferroptotic cell death in a pancreatic cancer cell line by increasing the concentration of intracellular ferrous iron and ROS production; it also exerts anti-tumor effects in *in vivo* experiments with less toxicity[141].

The combinatorial regimen using plant derivatives also expresses effective anticancer effects in pancreatic cancer cells by inducing ferroptosis; cotylenin A (a plant growth regulator) in combination with phenethyl isothiocyanate, an anti-carcinogenic compound, stimulated ferroptotic cell death in PANC-1 cells (Figure 2)[142]. Artesunate, an antimalarial agent, induced ferroptosis in Kras-activated pancreatic ductal adenocarcinoma cell lines driven by ROS generation and lysosomal iron-dependent cell death without affecting normal pancreatic cell lines[143]. Piperlongumine alone, as well as in combination with cotylenin A and sulfasalazine, generates ferroptotic death of pancreatic cancer cells [144].

Ferroptosis in GC

GC is a heterogeneous disease among GI cancers, with over 1 million new cases worldwide where surgery is the primary treatment to prevent progression[145]. The factors involved in PUFA biosynthesis play an essential role in inducing ferroptosis sensitivity in GC[72]. Apatinib, an anti-tumor agent, decreases the expression of GPX4 and results in apatinib-mediated ferroptotic cell death in GC cells [146]. GC cells resistant to sorafenib-induced ferroptosis treatment by silencing SIRT6, one of the sirtuin proteins that plays a vital role in the regulation of metabolism, DNA repair, and cancer development



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Figure 2 Regulation of ferroptosis in gastrointestinal tumors is schematically illustrated. The factors cotylenin A, phenethyl isothiocyanate, artesunate, and piperlongumine induce lipid peroxidation and thereby ferroptosis. Small molecules such as erastin, sorafenib, and sulfasalazine inhibit system Xc^- thereby inducing ferroptosis through glutathione peroxidase 4 (GPX4). Cisplatin, curcumin, and apatinib inhibit GPX4 thereby attenuating reactive oxygen species. Several inducers of ferroptosis such as RAS-selective lethal 3, FINO2, and FIN56 inhibit GPX4. Several synthetic and natural compounds regulate ferroptosis through hemoxygenase leading to iron overload. Transferrin receptor 1 mediates endocytosis of iron particles and facilitates ferroptosis. CDGSH iron sulfur domain 1, an iron chelator, blocks iron production and inhibits ferroptosis. Several compounds mediate ferritin degradation *via* ferritinophagy and modulate ferroptosis. CAPE: Caffeic acid phenethyl ester; CISD1: CDGSH iron sulfur domain 1; GSH: Glutathione; GSSG: Glutathione disulfide; HO-1: Heme oxygenase-1; PEITC: Phenethyl isothiocyanate; RSL3: Ras-selective lethal 3; ROS: Reactive oxygen species; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; TFR1: Transferrin receptor 1.

and is primarily located in the cell nucleus. SIRT6 sensitizes GC cells to sorafenib-induced ferroptosis by the Keap1/Nrf2/GPX4 signaling pathway[125,147]. The ingredient from the Chinese medicine tanshinone IIA, isolated from the rhizome of *Saliva miltiorrhiza Bunge*, exhibits an anticancer effect on GC cells by inducing ferroptosis and downregulating p53-mediated *SLC7A11*[148].

Ferroptosis in esophageal cancer

Targeting ferroptosis will provide new avenues for esophageal cancer diagnostics and treatment strategies. Sulfasalazine, a ferroptotic inducer, inhibits the progression of esophageal cancer, and plant-derived compounds such as oridonin, a diterpenoid, have also been reported to stimulate ferroptotic cell death in esophageal cancer cells[149].

FERROPTOSIS AND LINK TO OTHER RCD PATHWAYS

Ferroptosis is intricately intertwined with other forms of cell death through various iron and lipid peroxidation proteins and several transcription factors. However, molecular mechanisms underlying the role of other forms of RCD remain poorly understood. For example, *TP53*, a critical mediator of tumor suppressive response involved in apoptosis, ferroptosis, and antioxidant response machinery like Nrf2, has a significant role in ferroptosis as well as autophagy. The molecular mechanisms associated with other forms of RCD pathways and ferroptosis are discussed herein.

Apoptosis and ferroptosis: Molecular switch

Numerous studies have reported the interconnection between apoptosis and ferroptosis. Apoptosis, typically through p53, induces cell cycle arrest, thereby preventing tumorigenesis; likewise, *TP53* is known to sensitize cells to ferroptosis, leading to a reduced tumor burden[150]. The widely known ferroptosis inducer erastin has the potential to induce the unfolded protein response and promote p53 expression through apoptotic markers PUMA, CHOP, and TRAIL, and TRAIL-dependent apoptosis implies an augmented link between apoptosis and ferroptosis[151]. A similar study on a metal-

encapsulated p53 plasmid construct by Zheng *et al*[152] was found to release ions of iron, instigating the Fenton reaction and leading to ROS oxyradical overload, thereby leading to ferroptosis-dependent apoptosis in the liver. This consequently reduced the tumor burden and prevented metastasis in mice. An imbalance in the ferroptotic process is implicated in severely hindering apoptosis induction; for example, cancer cells subjected to ferroptosis by cysteine starvation were found to have reduced GSH levels but failed to induce caspase activation, which is seminal in apoptosis[153].

Autophagy-dependent ferroptosis

Autophagy-dependent ferroptosis is putatively ferritinophagy; under an excessive Fe^{2+} milieu, ferritin degradation is mediated by Atg5, an autophagy regulator protein. Ferritin is a seminal protein complex with light and heavy chain polypeptides (ferritin light chain 1 and ferritin heavy chain 2) predominantly controlling iron metabolism. Atg5 and Atg7 knockdown is implicated in preventing erastin-induced ferroptosis, facilitating tumorigenesis[154]. Similarly, BECN1 or Atg6 is known to induce ferroptosis by regulating glutamine and cysteine and inhibiting system Xc^- through the BECN1-*SLC7A11* complex in cancer cells. Additionally, studies have reported BECN1 facilitates lipid peroxidation through malondialdehyde (MDA) stress modulation[155]. Observations from our laboratory demonstrated that Eupatilin exhibits anticancer effects in part through regulation of autophagy-mediated ferroptosis (data not shown).

In summary, while apoptosis, ferroptosis, and autophagy are all different cellular pathways, they can be linked in the sense that autophagy can play dual roles by promoting apoptosis and protecting against ferroptosis. In contrast, both apoptosis and ferroptosis are forms of RCD mediated by different enzymes and signals that manifest different morphological outcomes.

THERAPEUTIC ASPECTS OF FERROPTOSIS

The listed drugs have potential therapeutic applications and have been reported to regulate ferroptosis (Table 2)[156-164].

Although conventional drugs are implicated in inducing or modulating ferroptosis, resistance to these drugs supersedes the benefits. Therefore, the identification of compounds with neutral toxicity profiles and natural origins has garnered tremendous attention in ferroptosis and iron metabolism. The section below briefly discussed the role of natural compounds in ferroptosis.

Formasanin C (FC) is known to induce ferroptosis in p53-null and p53 wildtype cellular phenotypes of HCC, and FC treatment increases the mitochondrial morphology and membrane potential in HepG2 cells, as a hallmark of cells undergoing ferroptosis[165]. FC and cisplatin synergistic treatment is known to induce ferritinophagy and enhance therapeutic potential of cisplatin. Similarly, gallic acid (GA) is known to prompt lipid peroxidation and ferroptosis. Upon exposure to an Fe^{2+} chelator, GA activity is suppressed, which in part signifies its role in ferroptosis. GA exhibited anti-tumor effects in colorectal cancer by deterring GPX4 and elevating MDA expression[166]. Celastrol, a tri-terpenoid is reported to induce ROS production, thereby promoting ferroptosis in liver cancer cells. Structural protein activity revealed that celastrol directly binds to multiple orthologues of PDXs. PDX knockdown in turn elevates ROS production, ensuing ferroptosis[167]. Chen *et al*[168] reported that curcumin could induce ferroptosis in colorectal cancer by modulating expression of key ferroptotic markers Fer1, *SLC7A11*, GSH, MDA, and ROS through PI3K/mTOR pathway.

The mechanisms orchestrating ferroptosis has been the subject of interest in several cancers. Many investigators have contributed to identifying key molecules that regulate ferroptosis in GI tumors[169]. The above findings are helpful in understanding the mechanism of many synthetic and natural compounds in inducing iron-dependent cell death in GI cancers. There are ample avenues to further elucidate the mode of action and mechanistic aspects of how natural compounds could be synergistically used to induce ferroptosis.

CONCLUSION

The increasing incidence and mortality imposed by GI cancers, a dangerous malignancy, warrants novel therapeutic strategies. Ferroptosis, a form of non-apoptotic RCD, has been found to play a significant role in regulating the progression of GI cancers. This review delineated the major regulatory mechanisms involved in ferroptosis for better understanding to create a new opportunity for diagnosis and therapeutic intervention. The involvement of ferroptosis-associated factors and the effect of several drugs, including the first discovered ferroptotic inducers erastin, sorafenib, cisplatin, artesunate, piperlongumine, haloperidol, baicalein, bromelain, and saponins have been found to induce ferroptotic cancer cell death in GI cancers. Ferroptotic inducers synergized with various anticancer drugs in clinical trials have demonstrated effective therapeutic results in GI cancers. Thus, inducing ferroptosis may have significant potential for treating GI cancers and related malignancies. Overall, this review provides insights into the regulatory mechanisms involved in ferroptotic cell death for development of novel

Table 2 Conventional drugs and their mode of action in ferroptosis

GI cancer	Drugs	Pathway involved	Mode of action	Ref.
Colorectal cancer	Paclitaxel	p53, *SLC7A11*	Lipid peroxidation by suppressing GPX4 expression	[156]
	5-fluorouracil	Lipocalin2 (LCN2), xCT	Stimulates GPX4 expression by diminishing circulating iron levels	[157]
	Cisplatin	Ferrostatin-1	Conjugates with GPX and GSH modulation	[158]
	Cetuximab	GSH, GPX4, HO-1, *SLC7A11*, KRAS, RSL3	Through β-elemene synergism by inducing iron dependent ROS, GSH accumulation, and EMT regulation	[159]
	Dihydro-artemisinin (DAT)	Iron metabolism, GPX4	Sensitizes cells to ferroptosis by iron overload and lysosomal degradation	[160]
Hepatocellular carcinoma	Artesunate	Ferritin	Elevates lysosomal degradation partially through autophagy along with sorafenib; it induces cathepsin activation	[161]
	Sorafenib	Nrf2	Through metallothionein-1 activation (MT-1G–Nrf2) axis	[162,125]
	Deferoxamine	Iron metabolism	Through peroxidation and iron storage depletion	[163]
Gastric cancer	Cisplatin	Nrf2/Keap1-xCT	Through transcription factor ATF3 overexpression	[164]

ATF3: Activating transcription factor 3; EMT: Epithelial-mesenchymal transition; GI: Gastrointestinal; GPX4: Glutathione peroxidase 4; GSH: Glutathione; HO-1: Heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2–related factor 2; ROS: Reactive oxygen species; RSL3: Ras-selective lethal 3; *SLC7A11:* Solute carrier family 7 member 11.

therapeutic strategies. The mechanism of ferroptosis with other RCD, such as autophagy and apoptosis, to induce cancer cell death may also provide new development in the therapeutic aspects of treating GI tumors. Therefore, further investigation of ferroptosis in GI cancers will improve the prognosis and therapeutic aspects of GI cancers.

FOOTNOTES

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REVIEW

Updates on global epidemiology, risk and prognostic factors of gastric cancer

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Abstract

Gastric cancer (GC) is defined as the primary epithelial malignancy derived from the stomach, and it is a complicated and heterogeneous disease with multiple risk factors. Despite its overall declining trend of incidence and mortality in various countries over the past few decades, GC remains the fifth most common malignancy and the fourth leading cause of cancer-related death globally. Although the global burden of GC has shown a significant downward trend, it remains severe in certain areas, such as Asia. GC ranks third in incidence and mortality among all cancer types in China, and it accounts for nearly 44.0% and 48.6% of new GC cases and GC-related deaths in the world, respectively. The regional differences in GC incidence and mortality are obvious, and annual new cases and deaths are increasing rapidly in some developing regions. Therefore, early preventive and screening strategies for GC are urgently needed. The clinical efficacies of conventional treatments for GC are limited, and the developing understanding of GC pathogenesis has increased the demand for new therapeutic regimens, including immune checkpoint inhibitors, cell immunotherapy and cancer vaccines. The present review describes the epidemiology of GC worldwide, especially in China, summarizes its risk and prognostic factors, and focuses on novel immunotherapies to develop therapeutic strategies for the management of GC patients.

Key Words: Gastric cancer; Epidemiology; Risk factors; Prognosis; Treatment;

Immunotherapy

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Core Tip: As a malignant disease with decreasing trends in incidence and mortality, gastric cancer (GC) remains a public health issue worldwide. Various risk factors have been suggested, and the prognosis of GC is related to various factors, such as tumor location, lymph node metastasis, gene polymorphisms and therapeutic strategies. Therefore, novel treatments have been proposed, and immunotherapy has attracted more attention. The present review discusses the epidemiology, risk and prognostic factors of GC with a focus on immunotherapy to better inform the management of GC patients.

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INTRODUCTION

Despite its global declines in incidence and mortality over the past several decades, gastric cancer (GC) remains responsible for 1.089 million new cancer cases and 0.769 million deaths in 2020 worldwide, which makes it the fifth-most common malignancy and the fourth leading cause of cancer-related deaths, according to Global Cancer Statistics (GLOBOCAN) 2020[1]. The global age-standardized incidence and mortality rates for GC were 11.1/100000 and 7.7/100000 in 2020, with wide geographical variations[2]. These data highlight that GC remains a major global health challenge. As a primary epithelial malignancy derived from the stomach, the initiation of GC is a multistage process and is generally associated with various risk factors[3], and some elements are related to its prognosis and survival[4,5]. Thanks to advances in preventative, screening and therapeutic strategies, the incidence and mortality of GC has been decreasing gradually worldwide. However, certain challenges still exist in the management of GC, such as the clinical applications of surgical treatment and chemotherapy. Recent immunotherapy for GC has drawn much attention, and it improved the current therapeutic situation. The present review describes the epidemiology of GC in different regions in the world, especially in China, summarizes its risk and prognostic factors, and focuses on new immunotherapies to develop therapeutic strategies for the management of GC patients.

GLOBAL EPIDEMIOLOGY OF GC

Incidence and mortality rates of GC around the world

The GLOBOCAN 2020 database (https://gco.iarc.fr/)[1] estimated that there were 1089103 newly diagnosed GC cases in 185 countries, with GC ranking fifth in incidence among all cancer types globally (Figure 1A). According to the anatomical locations, GC is classified as cardia GC and noncardia GC with different epidemiological profiles[1], and noncardia GC is the most common subtype[6]. There is a significant difference in GC incidence in sex distribution, and the age-standardized incidence rate (ASIR) is 15.8/100000 in males and 7.0/100000 in females, which indicates that GC incidence is approximately 2-fold higher in males than females[1]. GC incidence ranked fourth in males and seventh in females among all cancer types[1] (Figure 1A). Geographic variations in the ASIR in GC are up to 1- to 4-fold worldwide[7]. The ASIR is highest in Asia (14.3/100000), followed by Latin America and the Caribbean, Europe and Oceania, and it is lowest in Africa and North America[7] (Figure 2A). Most GC cases are diagnosed in countries with a high and very high human development index (HDI), such as eastern and southeastern Asian countries, central and eastern European and South American countries, and the ASIRs in these countries were higher than countries with a medium and low HDI[2] (Figure 2B). The five countries with the highest ASIRs in Asia were Mongolia (32.5/100000), Japan (31.6/100000), Republic of Korea (27.9/100000), Tajikistan (23.4/100000) and China (20.6/100000) (Figure 3A), which indicated that greater than 69% of the total GC cases in 2020 occurred in eastern and south-central Asia. In addition, Figure 3B and C show the detailed information about the male and female ASIRs in Asia.

GC is the fourth most common cause of mortality among all cancer types, followed by lung, colorectal and liver cancers[1] (Figure 1B). A total of 768793 deaths were estimated to be related to GC, with an overall mortality of 7.7/100000 globally, and sex differences exist with males being twice as likely as females to exhibit the disease (Figure 1B)[1]. The age-standardized mortality rate (ASMR) of GC was

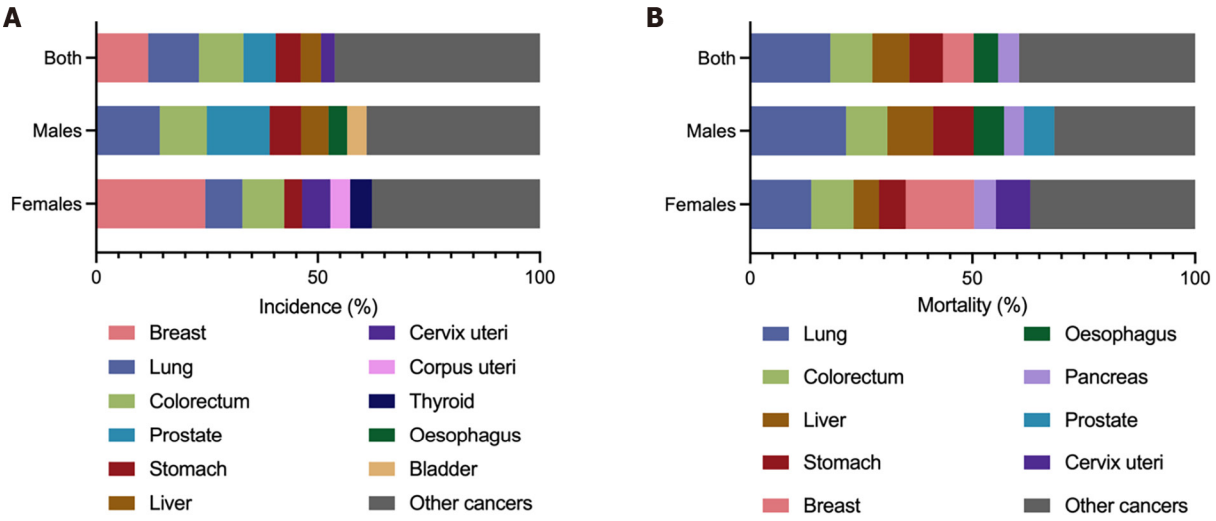


Figure 1 The composition of incidences and mortalities of all cancer types in 2020 globally. A: The composition of incidences of all cancer types in 2020 globally; B: The composition of mortalities of all cancer types in 2020 globally. Bar plots show the composition of incidences or mortalities of all cancer types in both sexes, males and females, respectively. Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

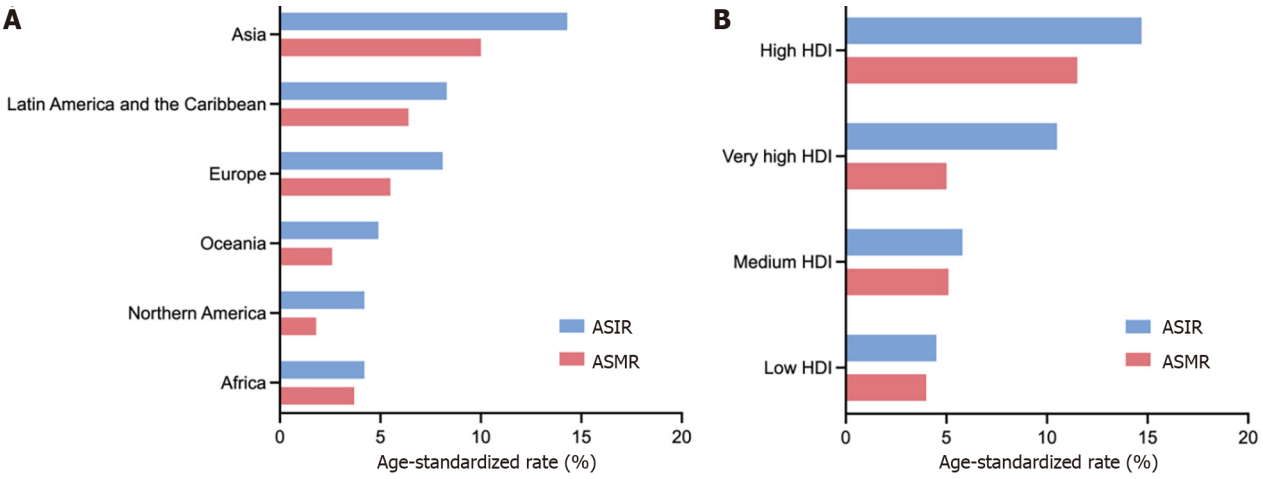


Figure 2 Age-standardized incidence and mortality rates of gastric cancer in 2020 worldwide. A: Age-standardized incidence and mortality rates of gastric cancer (GC) in 2020 in the five continents; B: Age-standardized incidence and mortality rates of GC in countries classified by human development index in 2020 worldwide. ASIR: Age-standardized incidence rate (1/100000); ASMR: Age-standardized mortality rate (1/100000); HDI: Human development index. Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

highest in Asia (10.0/100000) (Figure 2A). Countries with a very high HDI have higher mortality rates, and countries with a medium and low HDI have lower mortality rates, which is consistent with GC incidence (Figure 2B). The five Asian countries with the highest ASMRs were Mongolia (24.6/100000), Tajikistan (19.7/100000), China (15.9/100000), Bhutan (15.9/100000) and Kyrgyzstan (15.7/100000) (Figure 3D), and the male and female ASMRs in Asia are shown in Figure 3E and F, respectively. Mongolia has the highest incidence and mortality rates, primarily due to the lack of endoscopy and professional endoscopists[8].

The overall GC incidence and mortality rates have steadily declined in most countries during the past several decades, with evident decreases in males and females[1,9-13], as preventative, screening and therapeutic programs have been implemented worldwide[14-16]. For example, the ASIR of GC in Korea decreased significantly from 2011 (ASIR 43.0) to 2019 (ASIR 29.6)[17]. Similar to most other cancers, GC is generally rare in adults aged < 50 years, and its incidence increases with aging[9,10]. However, GC incidence has presented an increasing trend in younger generations (below age 50 years) in high- and low-incidence areas, such as the United States and the United Kingdom, compared to older individuals who exhibited a decreasing trend in GC incidence[9,10,18]. One United States study reported a more

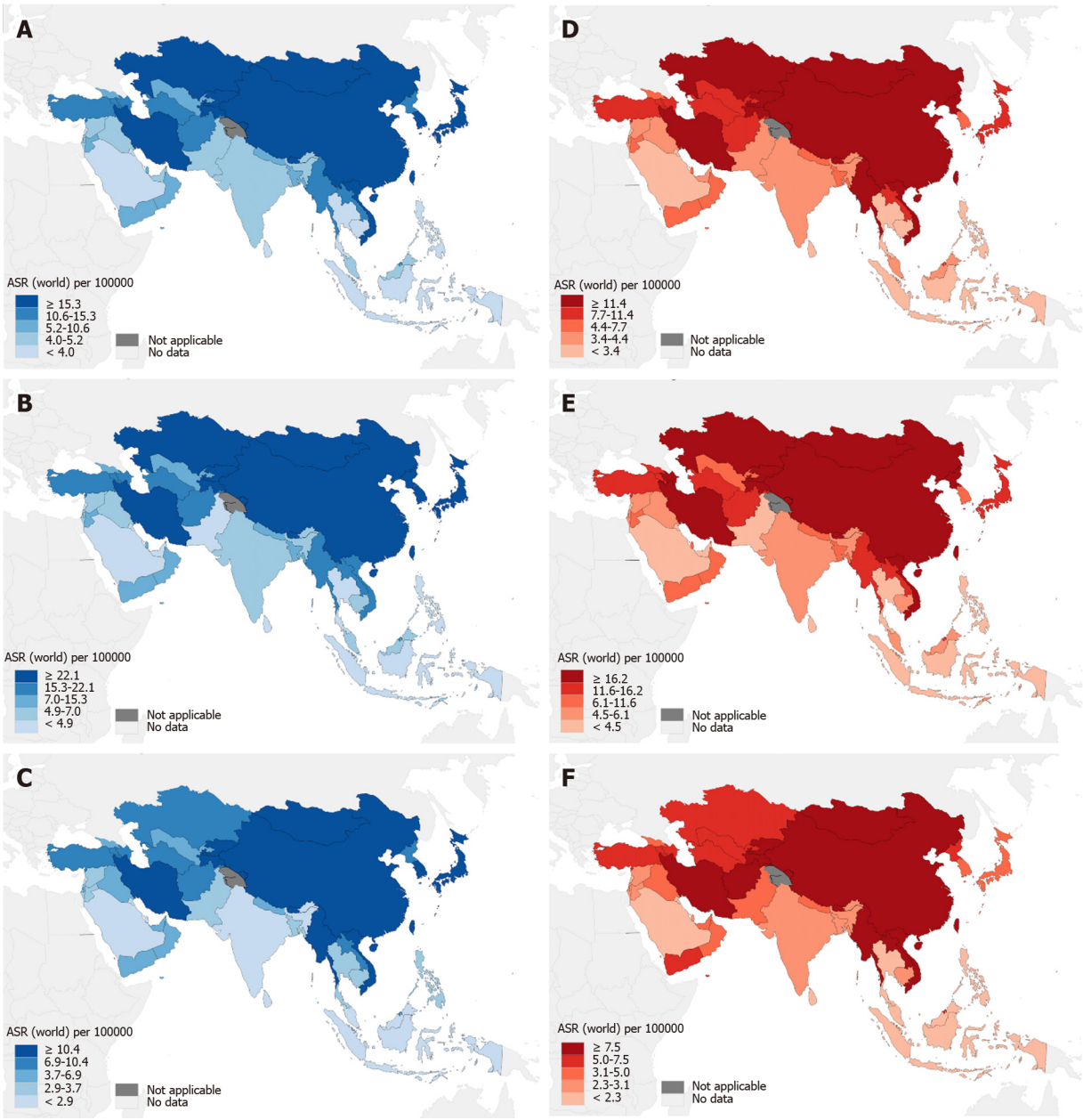


Figure 3 Estimated age-standardized incidence and mortality rates of gastric cancer in 2020 in Asian countries. A: Estimated age-standardized incidence rates of gastric cancer (GC) in 2020 in Asian countries; B: Estimated age-standardized incidence rates of GC in males in 2020 in Asian countries; C: Estimated age-standardized incidence rates of GC in females in 2020 in Asian countries; D: Estimated age-standardized mortality rates of GC in 2020 in Asian countries; E: Estimated age-standardized mortality rates of GC in males in 2020 in Asian countries; F: Estimated age-standardized mortality rates of GC in females in 2020 in Asian countries. ASIR: Age-standardized incidence rate (1/100000); ASMR: Age-standardized mortality rate (1/100000). Citation: Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209-249. Copyright ©International Agency for Research on Cancer 2020. Published by International Agency for Research on Cancer[1].

pronounced increase in incidence in younger females than males and predicted that the overall incidence may no longer be decreasing, and the GC incidence in females may exceed males if this pattern continues[18]. The 5-year overall survival (OS) rate shows that GC survival has improved due to advances in diagnostic and therapeutic strategies, especially with early detection from national screening programs using endoscopic and/or radiographic methods[9,19]. For example, one study found that the 5-year survival rate of GC in Korea increased from 55.7% in 1999-2005 to 77% in 2013-2019[17], which is consistent with the previous cancer statistics in Korea in 2015[20]. However, GC maintains a high case fatality rate, and it is a main contributor to the global burden[21].

Epidemiology of GC in China

GC is also one of the major malignances in China. The ASIR and ASMR of GC were higher in 2012 (ASIR 22.06/100000, ASMR 15.16/100000) than 2016 (ASIR 17.59/100000, ASMR 12.30/100000), with a

decreasing trend from 2012 to 2016 and decreasing trends in males and females[22-26] (Figure 4). The National Cancer Center of China reported 396500 new GC cases and 288500 GC-leading deaths in China in 2016[26], and the ASIR and ASMR of GC ranked fifth (17.59/100000) and third (12.30/100000) among all cancer types, respectively[26,27]. The number of new GC cases in males was nearly 276300 (ASIR 25.14/100000), and GC-related deaths (200200, ASMR 17.77/100000) accounted for approximately 13% of all male cancer-related deaths. The number of new GC cases in females was approximately 120200 (ASIR 10.31/100000), and GC-related deaths (88400, ASMR 7.13/100000) accounted for approximately 10% of all female cancer-related deaths[26]. GLOBOCAN 2020 indicated 479000 new GC cases and 374000 GC-related deaths in China in 2020, which ranked third in incidence and mortality among all cancer types[28] and accounted for 44.0% and 48.6% of new GC cases and GC-related deaths worldwide, respectively[1]. Current data in China (https://gco.iarc.fr/) showed that the ASIR and ASMR of GC were greater than 2-fold in males (ASIR 29.5/100000, ASMR 22.8/100000) than females (ASIR 12.3/100000, ASMR 9.5/100000) in 2022, and more GC cases and deaths occurred in patients over 60 years. Notably, geographic variations also exist in different areas of China. Specifically, the ASIRs and ASMRs of GC in urban areas were higher than rural areas[26]. The ASIR and ASMR were highest in northwestern China (ASIR 25.8/100000, ASMR 18.1/100000), followed by eastern (ASIR 21.9/100000, ASMR 14.8/100000) and central (ASIR 18.7/100000, ASMR 13.8/100000) areas of China, and southern China had the lowest ASIR of 9.2/100000 and ASMR of 6.4/100000[26].

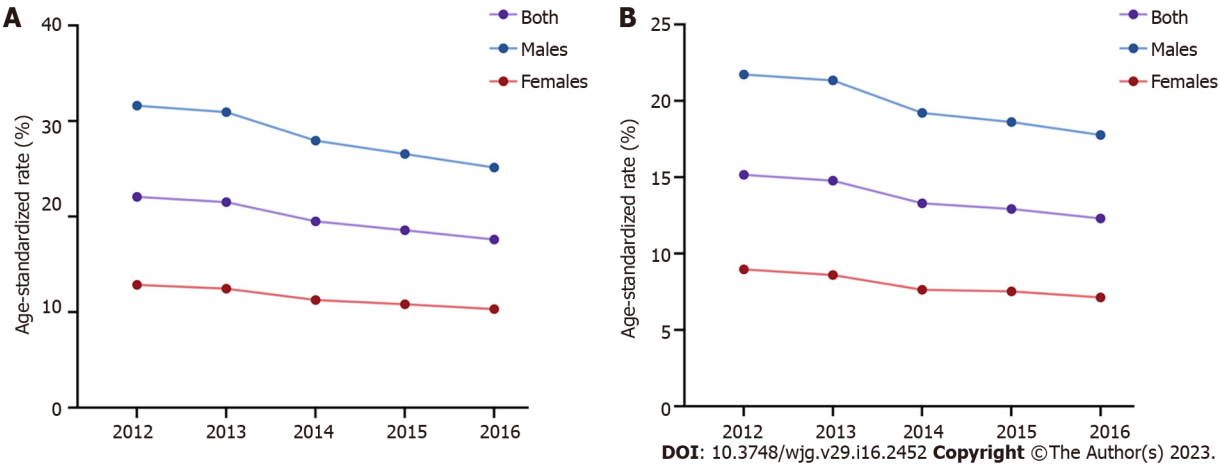
Because the early symptoms of GC are insidious, most GC patients are metastatic in the advanced stage at the time of diagnosis[6]. The proportion of GC patients diagnosed in the early stage was lower than advanced GC patients in China in the past[28]. However, the survival time is closely related to the stage at diagnosis for GC patients[19]. Thereafter, China proposed a series of guidelines aimed at improving GC screening, early detection and therapeutic strategies based on the popularity of gastrointestinal endoscopy, and the proportion of early GCs increased in recent years[28,29]. The 5-year OS rate of GC increased from 27.4% in 2003-2005 to 35.1% in 2012-2015 with an ascending trend in China[29], but it is significantly lower than Japan (81.0%) in 2004-2007[19] and South Korea (75.4%) in 2011-2015[20], which may be related to the different preventive, early screening, diagnostic and therapeutic strategies in individual countries[1].

RISK FACTORS

The pathogenesis of GC is complicated, and more attention should be given to individuals with higher GC risks for surveillance. Various factors may synthetically affect GC occurrence and development (Figure 5). Some risk factors are nonmodifiable, such as age, sex, race/ethnicity and genetics[6,28,30,31], and other controllable risk factors may include *Helicobacter pylori* (*H. pylori*) infection, gastrointestinal microbiota, obesity, unhealthy dietary habits and lifestyle, tobacco and alcohol consumption, and chemical, radiation or virus exposure[6,16,28,32-34]. Several relatively rare risk factors may also participate in GC pathogenesis, such as gastroesophageal reflux disease, gastric ulcer or previous gastric surgery[30].

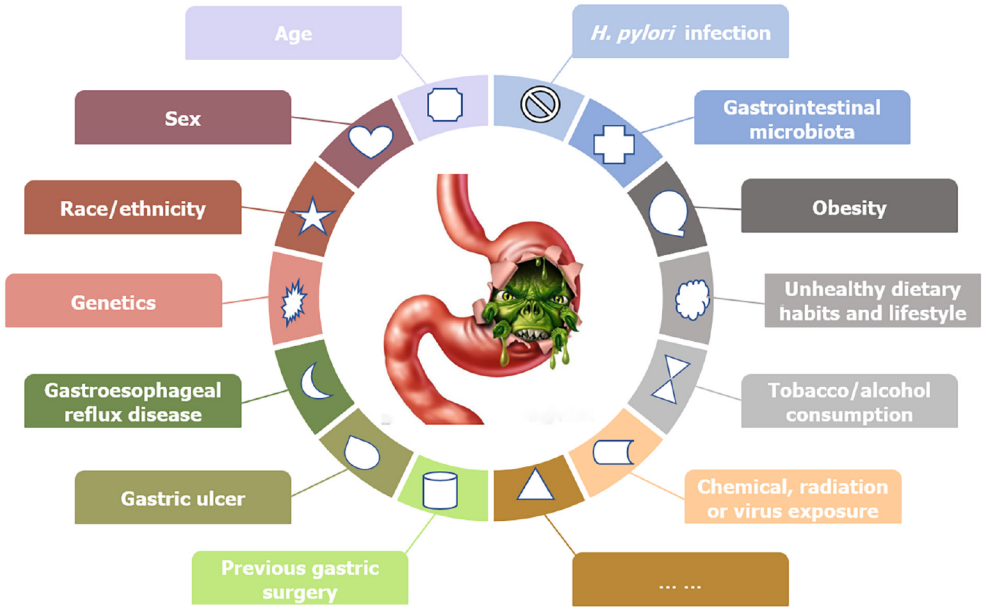
One study reported that 1.8% of GC cases occurred in individuals younger than 34 years, 38.6% occurred in adults between 35 and 64 years old, and 59.6% occurred in elderly individuals over 65 years from 2015 to 2019[35], with a median age at diagnosis of 68 years, which indicate that GC risk increases with aging. Sex differences exist in GC incidence, which is almost twofold higher in males than females [1,35]. These data suggest the protective effect of sex steroid hormones in GC pathogenesis[36]. Males tend to have higher risks of *H. pylori* infection than females, which may also lead to sex differences[37,38]. Approximately 10% of GC cases exhibited familial aggregation, which indicates that a family history of GC may be an independent risk factor[39,40]. A total of 1%-3% of GC patients may have germline mutations, and the underlying molecular mechanisms have not been fully clarified[41].

Chronic *H. pylori* infection is the major confirmed cause of GC, and it may be related to approximately 90% of noncardia GC cases[10,42-44]. *H. pylori* is a Gram-negative pathogenic bacterium and an indigenous member of the gastric microbiota[18,45]. The prevalence of *H. pylori* infection in adults exceeds 50% of the human population with regional variations globally[1,45,46]. *H. pylori* infection is easily acquired during childhood[47], and it is generally carried asymptomatically for a lifetime. Since 1994, *H. pylori* has been classified as a class I carcinogen by the World Health Organization. The long-term colonization of *H. pylori* in the gastric mucosa contributes to the development of various gastric diseases, such as persistent inflammation, chronic gastritis, gastric mucosal atrophy and intestinal metaplasia, with its different genes encoding virulence factors[45,48]. Chronic *H. pylori* infection also induces epigenetic and genetic changes in gastric epithelial cells, which suggests the genetic instability of these cells[48]. Therefore, *H. pylori* infection is etiologically related to GC, and the duration also predisposes individuals toward GC later in life[48]. Because *H. pylori* infection is closely related to noncardia GC, its eradication significantly decreased the incidence of noncardia GC[48]. However, *H. pylori* infection is only necessary, but not sufficient, in the pathogenesis of GC[48]. Although controversial[18], *H. pylori* screening and eradication has been proposed as a preventive strategy for GC[48]. Data from healthy asymptomatic infected Asians showed that eradicating *H. pylori* reduced GC



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Figure 4 Trends in age-standardized incidence and mortality rates of gastric cancer according to the National Central Cancer Registry of China in 2012-2016[22-26]. A: Trends in age-standardized incidence rates of gastric cancer (GC) according to the National Central Cancer Registry (NCCR) of China in 2012-2016; B: Trends in age-standardized mortality rates of GC according to the NCCR of China in 2012-2016.



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Figure 5 Summarization of risk factors of gastric cancer. *H. pylori*: *Helicobacter pylori*.

incidence[49], and population-based screening and eradication of *H. pylori* infection was cost-effective [50]. The efficacy of eradicating *H. pylori* to prevent GC also depends on other risk factors, such as the time of *H. pylori* eradication, intragastric acidity, resistance to antimicrobial agents and the compliance of infected patients[48,51,52]. In contrast, a similar screening strategy may not be economical and is generally unwarranted in some countries with low GC incidences[2]. Anderson *et al*[18] once indicated that population-based *H. pylori* eradication in the United States may raise certain unanswered questions about safety, efficacy, unanticipated consequences and failure to reduce the GC burden. Less than 5% of *H. pylori*-infected individuals may develop GC due to the genetics of *H. pylori* and the host, duration of *H. pylori* infection and certain environmental factors[1,53]. The efficacy of *H. pylori* eradication and its cost-effectiveness should be further investigated in different geographic regions[48].

With the progression of molecular biological technologies, such as next-generation sequencing, a series of studies examined the correlations between GC and gastric microbiota other than *H. pylori*[54, 55]. Ferreira *et al*[56] performed 16S rRNA gene sequencing analysis of gastric microbiota for 54 GC patients and 81 gastritis controls, and found that the gastric microbiota of GC had reduced microbial diversities, reduced abundance of *H. pylori*, and the increment of other microbial genera that were similar to intestinal microbiota; besides, the nitrosating functions and genotoxic potential were also increased. He *et al*[57] investigated the characteristics of the intestinal microbiota in fecal samples from GC patients and healthy individuals using 16S rRNA gene sequencing technology, and showed that the

relative abundances of *Faecalibacterium*, *Bifidobacterium*, *Subdoligranulum*, *Enterococcus*, *Streptococcus* and *Bacteroides* were closely associated with GC risk and occurrence. Aziz *et al*[58] found four types of microbial proteins in serum and tissue biopsy specimens of GC patients, including *Acinetobacter baumannii*, *Escherichia coli*, *Fusobacterium nucleatum* and *Bacteroides fragilis*, as well as *H. pylori*. Overall, microbiota-related GC studies indicated an alteration of the gastric microbiota during gastric carcinogenesis, which was distinct from patients with chronic gastritis and healthy individuals[59]. Therefore, it is necessary to further analyze the gastric microorganisms and explore the possible underlying pathogenesis of microbial dysbiosis in the progression to carcinoma to provide guidance for preventative, screening and therapeutic strategies for GC.

The diverse risk and protective factors of GC are dietary or lifestyle-related factors[60], and unhealthy dietary habits and lifestyle factors may account for 33%-50% of all GC cases[10,61]. Lower intake of fruit and vegetables, higher intake of salt or salted/processed food, and tobacco and alcohol consumption are GC risk factors[60,62,63]. For example, a 5 g/day increase in salt intake increased the risk of GC by 12% [53]. Excessive salt intake may destroy the gastric mucosa and increase DNA synthesis and cell proliferation to promote the development of GC[64], and excessive salt intake also acts synergistically with *H. pylori* to increase GC incidence[60,65,66]. However, no scientific evidence has confirmed a definite causal association between excessive salt intake and GC risk[60]. Tobacco consumption is also an important behavioral risk factor for GC and may increase GC risk by approximately 50% in males and 20% in females according to relevant data[32,67,68]. Tobacco consumption may induced chronic inflammation in the gastrointestinal tract, alter mucosal cell proliferation, promote immune dysfunction, and increase the risks of bacterial or viral infections, which leads to the carcinogenesis of GC[69]. Alcohol consumption also positively correlates with GC risk[69-71]. These two factors, tobacco and alcohol consumption, affect GC development independently in high-risk populations, and modification of these unhealthy choices may significantly reduce the incidence and mortality of GC[72]. In contrast, increased fresh fruit and vegetable consumption may inhibit GC development and reduce its risk[53]. Specifically, a systematic review and dose-response meta-analysis found that a 100 g/day increase in fruit consumption inversely associated with a 5% reduction in GC risk[53]. Another meta-analysis also indicated that the relationship between intake of citrus fruit and risk of cardia GC was statistically significant, and daily intake of 100 g citrus fruit reduced GC risk by 40%[73], which suggests that phytochemicals in fruit may have antioxidant effects, prevent or reduce DNA oxidation, and regulate cell proliferation and apoptosis[74]. The history of medication may affect GC pathogenesis, and the use of aspirin and other nonsteroidal anti-inflammatory drugs may lower GC risk[75].

In summary, various known and unknown factors may be related to GC risk, and understanding the underlying mechanisms will facilitate appropriate preventative and screening strategies to reduce GC incidence.

PROGNOSTIC FACTORS

Patient- and tumor-related prognostic factors

Although the incidence of GC in males is significantly higher than females[1], few studies have focused on sex differences in GC prognosis. The relationships between race, gene polymorphism and GC prognosis have been explored in many studies[76,77]. Current data show high GC incidences in Asian populations and better GC prognosis in Asian GC patients than Caucasian populations, even after controlling for other well-known prognostic factors[78-80]. However, whether differences exist due to different management strategies or distinct races and tumor biology is not clear. Increasing evidence indicates that genetic polymorphisms are associated with GC survival, and single nucleotide polymorphisms (SNPs) are novel biomarkers of cancer susceptibility, progression and prognosis[81,82]. For example, Gonzalez-Hormazabal *et al*[83] found that allele A carriers of IL-8 rs4073 were associated with lower OS in GC. The role of ERCC1 SNPs was also extensively studied in patients with gastrointestinal tumors receiving oxaliplatin-based chemotherapy, and ERCC1 rs11615 polymorphisms were closely associated with the clinical outcomes of GC[84]. Wang *et al*[85] showed that lncRNA H19 rs2839698 was also related to the OS of GC patients. However, more prospective studies with larger sample sizes are needed to verify the above conclusions and elucidate the underlying mechanisms of SNPs in GC prognosis.

The Lauren classification[86] is globally recognized as the classification system for GC. According to the Lauren classification[86], GC is classified into intestinal and diffuse types, and diffuse GC generally has a poor prognosis compared to intestinal GC[87-89]. The survival of GC is strongly related to the stage at diagnosis. For patients with early GC, the cancerous area is localized with no local or distant metastasis, and these patients generally have a better prognosis than patients with advanced GC[4]. The cancer site in advanced GC patients is not localized and generally accompanied by metastasis[90]. GC easily recurs even after surgical resection, and it generally has a poor prognosis[91]. Tumor size, depth of invasion, lymph node metastasis (LNM), and tumor-node-metastasis stage are important prognostic factors[5]. For example, the 5-year survival rate of patients with proximal GC is lower than patients with distal GC, which indicated the correlation of tumor location and GC prognosis[5]. Notably, a Chinese

study[92] recruited 611 patients with early GC and showed no significant difference in 5-year survival between mucosal and submucosal cancers. Notably, LNM may be a significant prognostic factor for early GC[92,93], and the 5-year survival rate may be twice as high in patients without LNM than patients with LNM[94].

Treatment-related prognostic factors

A variety of factors affect GC prognosis, including patient-related factors (gender, age, race), tumor-related factors (tumor location, histological type, depth of invasion, and metastasis) and treatment-related factors. We primarily focused on the prognostic factors related to GC treatment.

Surgery is the basis of GC treatment, and it is the only procedure that completely eradicates GC lesions[3,91,95]. Surgical options primarily include endoscopic mucosal resection, distal esophagectomy, subtotal gastrectomy or total gastrectomy[96]. The surgical strategies depend on the tumor location and invasion depth, and may vary in different institutions. For early GC with a low LNM risk, endoscopic treatment or surgery alone may be effective, and patients with advanced GC may benefit from broad lymph node dissection and multimodal therapies[97]. In terms of the extent of lymph node dissection (D), current data indicate that D2 lymph node dissection is the most recommended surgical procedure for advanced GC, and GC patients may have a higher 5-year survival rate after D2 lymph node dissection than after D1 dissection[98]. Accurate preoperative staging and resection evaluation are key factors in successful surgery for GC[95]. The precision medicine of surgical strategies for GC, by the concept of accelerated rehabilitation, includes minimally invasive surgery, precise operation of intraoperative fluorescence navigation and precise perioperative management[95]. Compared to traditional surgery, minimally invasive surgery, including laparoscopy and da Vinci robot-assisted surgery, may reduce surgical trauma and improve recovery after surgery[99]. Although no significant differences in postoperative outcomes were found between laparoscopic and robotic gastrectomies, robotic gastrectomy may require a longer surgical duration and greater financial cost, and is not superior to laparoscopic procedures in perioperative surgical outcomes[99]. Patients with advanced GC need lymph node dissection, which is difficult and complicated. Therefore, whether laparoscopy is suitable for these patients needs further evaluation[95].

Because most GC patients are in advanced stages at initial diagnosis, surgery alone may not be sufficient to treat this malignancy, and therapeutic methods other than surgery should also be performed to improve the survival of operable GC patients[95]. As another important conventional treatment, chemotherapy is classified as perioperative, neoadjuvant or adjuvant, and palliative chemotherapy[28]. The OS of GC patients was significantly improved with perioperative chemotherapy compared to GC patients after surgical resection alone, and perioperative chemotherapy also increased the OS compared to postoperative chemotherapy[100,101]. Specifically, there are several combined options for chemotherapy strategies[28,102,103], including neoadjuvant triple chemotherapy, such as docetaxel, oxaliplatin and S-1 (DOS) or Epirubicin, cisplatin and 5-fluorouracil (ECF), and double chemotherapy, such as capecitabine and oxaliplatin (XELOX), tegafur and cisplatin (SP) or 5-fluorouracil and oxaliplatin (FOLFOX). The ToGA trial[104] showed that trastuzumab in combination with chemotherapy improved survival for HER2-positive GC patients, and an anti-HER2 targeting strategy was proposed as a standard option for HER2-positive GC patients. For example, trastuzumab plus XELOX, trastuzumab in combination with capecitabine, or bevacizumab and trastuzumab combined with docetaxel, oxaliplatin and capecitabine achieved encouraging efficacy and safety for patients with HER2-positive advanced GC, and may be considered first-line treatments for these patients, but further evaluation is warranted[105-107]. However, trastuzumab combined with DOS showed less effectiveness than trastuzumab in combination with XELOX, and further investigation is ongoing[108]. The use of radiotherapy in GC treatment has become more common with the advancement of radiotherapy-related technology[109]. Radiotherapy is generally combined with surgery, chemotherapy, molecular targeted therapy or other treatments, and these combinations eventually benefit GC patients[28]. Radiotherapy may be used as an important adjuvant therapy in the perioperative period for patients with advanced operable GC, especially for some patients after D2 Lymphadenectomy, and it effectively improved progression-free survival time and reduced the local recurrence rate[109]. Notably, perioperative chemotherapy or postoperative chemotherapy plus radiotherapy are listed as preferred strategies in certain guidelines[30]. For GC patients in the early-stage with LNM, OS may be improved after the adoption of adjuvant chemotherapy and radiotherapy, but the benefit was less certain for adequately staged GC patients without LNM[110].

Immune-based therapy for GC

Although surgery, chemotherapy, molecular targeted therapy, radiotherapy or combined modality treatment improved the survival of GC patients[30,91], these treatments have limited efficacies in treating patients with advanced GC, and potential therapeutic strategies are urgently needed for these advanced GC patients. A number of recent studies[111,112] found that immune-based therapy for solid malignancies produced good results and significantly prolonged survival, and immunotherapy showed certain positive efficacies for GC patients compared to other traditional therapies[113-115], which may bring new hope to GC patients[116]. There are three main immunotherapeutic options for GC, including immune checkpoint inhibitors (ICIs), cellular immunotherapy and cancer vaccines.

As a co-suppressor molecule, the immune checkpoint regulates the survival, proliferation, differentiation or response to homologous antigens of T cells *via* the major histocompatibility complex-T-cell receptor, prevent excessive immune responses, and maintain the immune homeostasis in the human body[117,118]. Tumor cells in patients with malignant diseases could inhibit T cells then escape immune responses *via* the mechanisms described above[119]. Immunosuppressants are primarily used to reactivate the immune responses of T cells to tumor cells by blocking immune checkpoints or corresponding ligands/receptors with antibodies[120]. ICIs have been widely investigated[121], and these monoclonal antibodies[30] primarily target programmed cell death protein-1 (PD-1), programmed death ligand-1 (PD-L1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4). PD-1 is a co-inhibitory receptor that is primarily expressed on the surface of activated T cells, Treg cells and monocytes. As the ligand of PD-1, PD-L1 binds to the PD-1 receptor and induces the inhibition or apoptosis of related immune cells, which helps tumor cells escape immune responses[122]. Moreover, PD-L1 is overexpressed in advanced GC, and its expression may relate to the tumor size, depth of invasion and LNM in 25%-65% of GCs[122-124]. Furthermore, clinical trials indicated that anti-PD-1 therapy for GC patients may have certain effectiveness. The first randomized phase III study demonstrated that the anti-PD-1 monoclonal antibody, nivolumab, effectively improved survival of patients with advanced gastric or gastric-oesophageal junction cancer[125]. Another famous phase II clinical KEYNOTE-059 trial recruited 259 patients from 16 countries and showed that the PD-1/PD-L1 inhibitor pembrolizumab also had similar efficacy and safety for advanced GC patients[126]. Researchers in the phase III CheckMate-649 trial found that nivolumab combined with fluorouracil and platinum as first-line medications improved the OS of patients with advanced HER2-negative GC, gastroesophageal junction cancer or esophageal adenocarcinoma[127]. Mid-term analysis of the KEYNOTE-811 test showed that the combination of pembrolizumab and trastuzumab plus chemotherapy improved the overall response rate (ORR) of patients with advanced HER2-positive GC[128]. CTLA-4 is also found on the surface of activated T cells and may interact with B7-1/B7-2 on the surface of antigen-presenting cells, which results in inhibition of the CD28 signaling pathway, and plays a key role in T-cell activation[119,129]. A monoclonal anti-CTLA-4 antibody targets T-cell co-inhibitory receptor and reactivates T-cell anti-tumor immune activity [130]. However, preliminary studies indicated that some GC patients showed ineffectiveness or even remission after treatment with monoclonal anti-CTLA-4 antibodies, and the objective response rate with antibodies alone was not satisfactory because only one of 18 patients (5.5%) reached the primary endpoint[131]. Besides, the monoclonal anti-CTLA-4 antibody ipilimumab alone as the maintenance therapy did not show any improvements in FPS for patients with unresectable locally advanced or metastatic GC compared with the best supportive care in a phase II clinical trial[132]. One study, focused on the therapeutic efficacies of PD-1 and CTLA4 inhibitors, found that the ORR of patients with metastatic GC who received nivolumab plus ipilimumab was higher than patients who received only nivolumab[133]. However, Shitara *et al*[134] recently found that nivolumab plus ipilimumab did not improve the OS of HER2-negative patients compared to the chemotherapy regimen in advanced GC patients.

The 2022 National Comprehensive Cancer Network Guidelines proposed PD-1/PD-L1 inhibitors as first-line/second-line medications for GC treatment, but anti-CTLA4 immunotherapy was not suggested in GC treatment. However, clinical trials on anti-CTLA4 antibodies (ipilimumab and tremelimumab) are being performed[135]. Clinical trials on many other immunosuppressants, such as LAG3, Tim3, TIGIT and OX40, are also being performed. LAG3 and Tim3 are in phase I and II clinical trials, and TIGIT and OX40 are in the early research stage[136]. Although the toxicity of ICIs may limit their efficacy and clinical application[137], current evidence indicates that the combined modality of ICIs with other treatments may be more effective and applicable in GC, especially when combined with chemotherapy for advanced GC patients with drug resistance[116,138].

Cellular immunotherapy uses immune cytotoxic cells to recognize and attack tumor cells, and induce an effective antitumor response[138]. These immune cells may be expanded T cells and nature killer cells *in vitro* or gene-engineered T-cell receptor (TCR) T cells (TCR-Ts) and chimeric antigen receptor (CAR) T cells (CAR-Ts)[138,139]. TCR-T/CAR-T immunotherapies, as modified T-cell-based immunotherapeutic approaches, are targeted cellular therapies that take advantage of the cytotoxic potential of T cells to attack tumor cells in an antigen-specific manner[140]. For TCR-T immunotherapy, the target TCR genes that recognize specific tumor-associated antigens (TAAs) are transduced into peripheral blood T cells collected from patients, and these modified T cells are reinjected into the patients' circulation[138]. TAAs are presented by major histocompatibility complex-I (MHC-I) to TCR-Ts within the patient, and the combination of TAAs with TCR could activate these T cells to release cytokines and attack tumor cells[141]. TCR-Ts expressing KK-LC-1 (encoded by *CT83*) TCR recognized *CT83*$^+$ tumor cells *in vitro*, and KK-LC-1 is frequently expressed in human epithelial tumors, including GC with the highest expression[142]. NY-ESO-1 antibody positivity was also found in GC, which indicated that NY-ESO-1 may be another target for TCR-T immunotherapy of GC[143]. Although TCR-T immunotherapy is being applied in clinical treatment, there are still some challenges. For example, it is difficult to generate a universal TCR for immunotherapy because of the extreme polymorphism of the MHC locus [141]. To resolve these issues, CAR was developed based on antibody recognition specificities[141], and CAR-Ts are considered a promising class of antitumor treatment[144]. T cells are collected from autologous peripheral blood, genetically modified to produce specific CARs, namely CAR-Ts, then

reinjected into the patient's circulation[145-147]. CAR-Ts recognize and combine the specific antigen on the surface of tumor cells by the extracellular single-chain fragment variable domain, which results in the immobilization and clustering of CARs, the formation of nonclassical immune synapses and activation of CAR-Ts[141]. In contrast to MHC-restricted TCR-Ts, CAR-Ts are typically designed and engineered to recognize non-MHC cell surface proteins[141]. The density of the target antigen is particularly important in the modulation of CAR-T-cell signaling compared to TCR-Ts[141]. Only one kind of targeted tumor antigen may not be sufficient to obtain satisfying antitumor responses, and the expression of targeted antigen in other body cells inevitably results in transient and reversible harmful effects, such as cellular toxicity[144]. Several potential targets, such as NKG2D, FOLR1, HER2, MSLN and CLDN18.2, were found, and the real therapeutic efficacies need further evaluation[148-152]. Therefore, targeting GC-specific antigens remains a challenge, and the lack of truly GC-specific antigens limits the clinical application of CAR-T immunotherapy[144].

As an active antitumor immunotherapy, cancer vaccines are designed to enhance body immune function by inducing humoral and/or cellular immune responses[139,153]. Cancer vaccines primarily include autologous tumor cell vaccines, dendritic cell vaccines, peptide vaccines and genetically engineered vaccines[138]. Patients with gastroesophageal adenocarcinoma or untreated metastatic GC may have higher median OS rates after receiving the G17DT (Aphton) vaccine[154], and vaccination improved the OS of GC patients with good safety and tolerance, especially when combined with chemotherapy[116]. The immune responses (*e.g.*, changes in immune milieu and tumor immune escape mechanisms) to cancer vaccines may be rapidly and accurately monitored using molecular sequencing, artificial intelligence or cellular engineering, which could optimize the design of cancer vaccines and facilitate their clinical application[155].

CONCLUSION

The incidence and mortality of GC have shown a downward trend worldwide, which suggests that GC may become a rare disease in the future[16]. However, GC incidence and mortality rank fifth and fourth, respectively, among all cancer types worldwide, and it remains a major health challenge[16]. With regard to the identified GC risk factors, such as *H. pylori* infection and unhealthy dietary habits and lifestyle, preventive strategies could effectively reduce GC incidence. Therefore, more attention should be given to individuals at higher risk, and unified guidelines for GC surveillance should be established. Due to the different staging and therapeutic strategies used in different regions, the prognosis of GC patients varies greatly. Most GC cases are found in advanced stages at diagnosis, which limits the clinical application and efficacy of surgery[156]. Although chemotherapy has significantly improved the prognosis of advanced GC patients, enormous challenges remain, such as drug resistance and toxicity[107]. With the emergence and promising development of immunotherapy, its clinical application and efficacy have been evaluated in GC patients, especially in advanced GC patients. However, due to the complicated tumor microenvironment and the complex interactions between the immune system and tumor cells, more clinical trials on immunotherapy are needed to verify their efficacy and safety in GC patients.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Older adults with acute severe ulcerative colitis have similar steroid non-response and colectomy rates as younger adults

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Abstract

BACKGROUND

There is paucity of data on outcomes of acute severe ulcerative colitis (ASUC) in older adults (≥ 60 years of age).

AIM

To assess steroid non-response rates during the index admission for ASUC in older adults. Secondary outcomes were response to medical rescue therapy and colectomy rates; at index admission, 3 and 12 mo.

METHODS

This retrospective multicentre cohort study included ASUC admissions who received intravenous steroids between January 2013 and July 2020 at two tertiary hospitals. Electronic medical records were reviewed to collect clinical, biochemical, and endoscopic data. A modified Poisson regression model was used for analysis.

RESULTS

Of 226 ASUC episodes, 45 (19.9%) occurred in patients ≥ 60 years of age. Steroid non-response rates were comparable in older adults and patients < 60 years of age [19 (42.2%) *vs* 85 (47%), P = 0.618, crude risk ratio (RR) = 0.89 [95% confidence interval (CI): 0.61-1.30], adjusted RR = 0.99 (0.44-2.21). Rates of response to medical rescue therapy in older adults was comparable to the younger cohort [76.5% *vs* 85.7%, P = 0.46, crude RR = 0.89 (0.67-1.17)]. Index admission colectomy [13.3% *vs* 10.5%, P = 0.598, crude RR = 1.27 (0.53-2.99), adjusted RR = 1.43 (0.34-

6.06)], colectomy at 3 mo [20% *vs* 16.6%, *P* = 0.66, crude RR = 1.18 (0.61-2.3), adjusted RR = 1.31 (0.32-0.53)] and colectomy at 12 mo [20% *vs* 23.2%, *P* = 0.682, crude RR = 0.85 (0.45-1.57), adjusted RR = 1.21 (0.29-4.97)], were similar between the two groups.

CONCLUSION

In older adults with ASUC, the steroid non-response rate, response to medical rescue therapy, and colectomy rate at index admission, 3 and 12 mo is similar to patients less than 60 years of age.

Key Words: Elderly; Ulcerative colitis; Acute severe ulcerative colitis; Colectomy; Rescue therapy; Infliximab

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Core Tip: This is a retrospective study to assess the outcomes of older adults (≥ 60 years of age) hospitalised with acute severe ulcerative colitis (ASUC) as per Truelove and Witts' criteria. We identified 45 episodes of ASUC in older adults and compared outcomes with 181 episodes of ASUC in patients < 60 years of age. Older adults with ASUC have similar steroid non-response rate, response to medical rescue therapy and colectomy rates up to 12 mo from index admission, when compared to patients less than 60 years of age.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing-remitting, inflammatory disorder of the colon, resulting from numerous factors including genetic predisposition, environmental triggers, and gut microbiota[1,2]. Acute severe UC (ASUC), as defined by the Truelove and Witts criteria, occurs in 10%-25% at diagnosis and 20%-30% during the disease course of UC[3-5]. Intravenous corticosteroids (IVCS) remain the first-line therapy for ASUC, however infliximab (IFX) and ciclosporin (CsA) have been used as medical rescue therapy for those who are steroid-refractory[6-9].

Up to 20% of patients with UC have late-onset disease with their first flare occurring after the age of 60[10,11]. The basic principles of management of ASUC in older adults do not differ from younger patients[12]. However, there are unique challenges in managing inflammatory bowel disease (IBD) in older adults, including delay in diagnosis, misdiagnosis, and variable clinical presentations. Older adults may suffer from comorbidities, polypharmacy, complex drug-drug interactions, cognitive dysfunction, post-surgical complications, as well as social factors, which increase complexity in management of older adults with ASUC[13-17]. Studies have demonstrated higher treatment failure rates in elderly IBD patients who are commenced on their first anti-tumour necrosis factor agent[18]. In the setting of these factors, management decisions need to be patient-centred and individualised to minimise morbidity and mortality for older adults with ASUC.

Advanced age has not been shown to predict outcomes in ASUC[19]. However, in routine clinical practice, age is an important factor which is taken into consideration in the decision-making algorithm. As older adults are generally excluded from clinical trials, management decisions for these patients are often made by extrapolating data from a younger cohort of patients[11]. Moreover, short and long-term outcomes of ASUC in this cohort of patients are not well described. The primary outcome of the study was to assess steroid non-response rates during the index admission for ASUC in older adults. The secondary outcomes were response to medical rescue therapy and colectomy rates at index admission, 3 and 12 mo.

MATERIALS AND METHODS

Study population

All consecutive admissions with a diagnosis of UC at two tertiary Australian hospitals, from January 2013 to July 2020 at Gold Coast University Hospital and from January 2018 to July 2020 at Logan

Hospital, were identified using international classification of disease (ICD-10) codes (K51). Retrospective analysis identified adult patients (≥ 18 years of age) admitted for management of ASUC, as identified by Truelove and Witts criteria[3] (Figure 1). The study was approved by the Gold Coast Health Service Human Research Ethics Committee (Ref: LNR/2020/QGC/67173).

Inclusion was limited to patients with ASUC who received at least 3-5 d of IVCS (either hydrocortisone 400 mg/d or methylprednisolone 60 mg/d). Patients with a diagnosis of Crohn's disease or positive stool cultures for other enteric pathogens were excluded. Patients with superimposed Clostridium difficile or cytomegalovirus infection were included in the final analysis. Demographic, clinical and laboratory results were collected. Endoscopic data was collected from procedure reports and images, and scored based on the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) score[20].

Therapeutic management

All patients received IVCS as per international guidelines[21]. The Oxford criteria was used to determine failure of IVCS therapy after 3-5 d[22]. Patients received IFX or CsA for medical rescue therapy at their treating physician's preference. The standard dose IFX induction strategy utilised was 5 mg/kg at week 0, 2 and 6. Accelerated dose of IFX was defined as 10 mg/kg on day 0 followed by 5 mg/kg at week 2 and 6. The dose of IFX was determined by the treating physician based on clinical assessment of disease severity. CsA was dosed at 2 mg/kg body weight with a target trough level of 200-300 ng/mL at 48 h. In patients responding to medical rescue therapy, maintenance therapy was based on disease severity and prior treatment history as per the treating physician's discretion.

Definitions

UC: The diagnosis of UC was based on standard clinical, endoscopic, and histological criteria[23].

ASUC: The diagnosis of ASUC was based on Truelove and Witts criteria; defined as ≥ 6 bloody stool motions per day and one or more of the following: Haemoglobin < 10.5 g/dL, erythrocyte sedimentation rate ≥ 30 mm/hr or C-reactive protein ≥ 30 mg/L, temperature ≥ 37.8 °C, or heart rate ≥ 90 beats/min[3].

Disease extent: The maximum endoscopic extent at index colonoscopy according to the Montreal classification[24]. In patients with ASUC as their first presentation of disease, the extent was determined from the first available colonoscopy after discharge, or the surgical specimen if they underwent colectomy.

Older adults with ASUC: ASUC occurring in patients ≥ 60 years of age (irrespective of the age at diagnosis of UC).

Endoscopic severity: Defined by the UCEIS. The score (0-8) is calculated by the sum of three descriptors: Vascular pattern (scored 0-2), bleeding (scored 0-3), and erosions/ulcers (scored 0-3). It is assessed at the most severely affected area on flexible sigmoidoscopy[20].

Steroid non-response: Defined as failure to respond to IVCS as defined by the Oxford criteria[22], and receiving either medical or surgical rescue therapy.

IFX dosing: Standard dose strategy was defined as IFX 5 mg/kg at week 0, 2 and 6. Accelerated dose was defined as IFX 10 mg/kg on day 0 followed by 5 mg/kg at week 2 and 6.

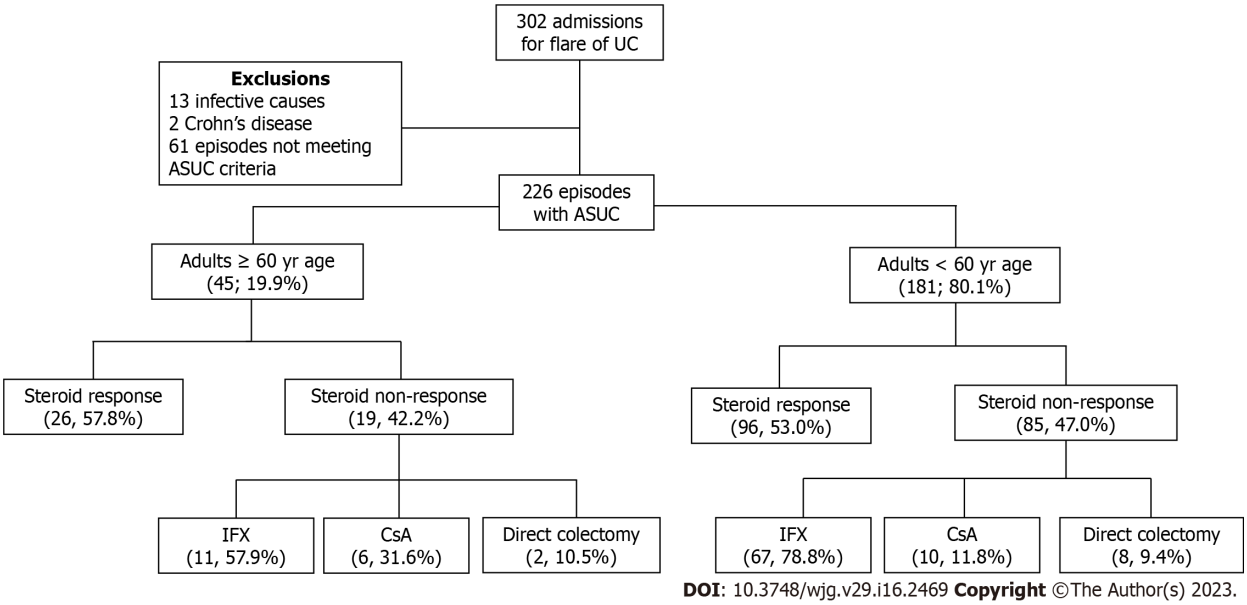
Responder to medical rescue therapy: Defined as the patient being discharged from hospital on medical therapy after receiving inpatient medical rescue therapy, and avoiding colectomy during the admission.

Outcomes

The primary outcome was to assess steroid non-response rates during the index admission for ASUC in older adults. The secondary outcomes were response to medical rescue therapy and colectomy rates at index admission, 3 and 12 mo.

Statistical analysis

Descriptive statistics were used to describe the study cohort. Results were reported as median with interquartile range (IQR) for continuous variables, and frequencies with percentages for categorical variables. For comparison of variables, Fisher's exact or Chi-square tests were used for categorical variables, and Wilcoxon Ranksum test for continuous variables. Continuous data was tested for normality using the Shapiro-Wilk test and a two-tailed P value of < 0.05 was considered statistically significant. A modified Poisson regression model was used to estimate risk differences (RDs) and RRs to evaluate the difference in clinical outcomes between the two groups. Kaplan-Meier plots and the Cox proportional hazards regression model were also used. A log-rank test was used to compare the curves of the Kaplan-Meier plots. Multiple imputations were performed to account for missing covariates. All analysis was performed using Stata15 (StataCorp LLC, College Station, Texas).



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Figure 1 Patient flow diagram. UC: Ulcerative colitis; ASUC: Acute severe ulcerative colitis; IFX: Infliximab; CsA: Ciclosporin.

RESULTS

A total of 302 admissions for UC who received IVCS were identified, of which 76 were excluded. 226 episodes of ASUC were included in the analysis. 45 (19.9%) episodes of ASUC in older adults ≥ 60 years of age and 181 (80.1%) episodes in younger adults were identified (Figure 1). Median age of disease onset was 66.5 (IQR: 59-76) *vs* 27 (IQR: 21-37), $P < 0.001$. Disease duration was similar between the two groups (2.5 *vs* 2 years, P 0.94). 33 out of 45 (73.3%) episodes had their first presentation of UC after the age of 60 years. Median Charlson Comorbidity Index in older adults was 3 (IQR: 2-4). Smoking status, albumin and platelet count at admission were significantly different between the two groups. Current immunomodulator use, biologic use and oral steroid use at admission were similar between the two groups. Clinical, endoscopic, and biochemical parameters are provided in Table 1. Summary of primary and secondary outcomes are shown in Table 2.

Primary outcome: Steroid non-response during the index admission for ASUC

Failure to IVCS therapy, as defined by the Oxford criteria[22], was similar between older and younger adults [19 (42.2%) *vs* 85 (47%), $P = 0.618$; crude RR = 0.89 (0.61-1.30), $P = 0.34$; adjusted RR = 0.99 (0.34-2.90), $P = 0.175$; odds ratio (OR) = 0.82 (0.43-1.58), $P = 0.344$; crude hazard ratio (HR) = 0.89 (0.556-1.455), $P = 0.674$]. In older adults, of the 19 episodes that failed IVCS, 17 (89.5%) episodes received medical rescue therapy (7 episodes IFX 5 mg/kg, 4 episodes IFX 10 mg/kg, 6 episodes CsA) and 2 (10.5%) patients proceeded directly to colectomy. Median time to initiation of rescue therapy was 4 d (IQR: 3-5 d). In patients < 60 years of age, of the 85 episodes that failed IVCS, 77 (90.6%) episodes received medical rescue therapy (45 episodes IFX 5 mg/kg, 22 episodes IFX 10 mg/kg, 10 episodes CsA) and 8 (9.4%) patients underwent direct colectomy. When the cut-off age was defined as 70 years, a significantly lower proportion of episodes failed IVCS [6/23 (26.1%) in ≥ 70 years *vs* 98/203 (48.3%) in < 70 years, $P = 0.049$; crude RD = -0.22 (-0.41 to -0.03); crude RR = 0.54 (0.27-1.09), $P = 0.034$; adjusted RR = 0.36 (0.08-1.49), $P = 0.897$; crude OR = 0.378 (0.143-1.00), $P = 0.05$].

Secondary outcomes

Response to medical rescue therapy: In older adults, of the 17 episodes who received medical rescue therapy, 4 (23.5%) patients underwent colectomy during the index admission. In the younger cohort, of the 77 episodes who received medical rescue therapy, 10 (13%) patients underwent a colectomy during the index admission. The rates of response to medical rescue therapy in older adults were similar to the younger cohort [76.5% *vs* 85.7%, $P = 0.46$; crude RD = -0.092 (-0.31 to 0.12); crude RR = 0.89 (0.67-1.17), $P = 0.27$; crude OR = 0.54 (0.16-1.85)]. When the cut-off age was defined as 70 years, a lower proportion of episodes responded to medical rescue therapy [4/6 (66.7%) in ≥ 70 years *vs* 75/88 (85.2%) in < 70 years, $P = 0.243$; crude RD = -0.18 (-0.57 to 0.19); crude RR = 0.78 (0.44-1.38); crude OR = 0.34 (0.65-), $P = 0.24$].

Index admission colectomy: In older adults, 6 (13.3%) of 45 patients underwent colectomy during the index admission for ASUC compared to 19 (10.5%) of 191 patients in the younger cohort [crude RD = 0.028 (-0.08 to 0.13); crude RR = 1.27 (0.53-2.99), $P = 0.376$; adjusted RR = 1.43 (0.34-6.06), $P = 0.71$; crude OR = 1.31 (0.50-3.41); crude HR = 1.27 (0.47-3.39), $P = 0.608$]. When the cut-off age was defined as 70

Table 1 Baseline characteristics of patients in the two groups (≥ 60 years and < 60 years)

	≥ 60 yr (*n* = 45)	< 60 yr (*n* = 181)	*P* value
Female, *n* (%)	22 (48.9)	88 (48.6)	1
Median age (yr)	71 (63-77)	32 (24-42)	< 0.001[1]
Median disease duration (yr)	2.5 (0-5)	2 (0.1-6)	0.94[1]
Index presentation of UC as ASUC (*n*, %)	14 (31.1)	45 (24.9)	0.45
Median follow up post admission for ASUC (wk)	104 (20-160)	74 (30-168)	0.97[1]
Median symptom duration before admission (d)	14 (7-24)	14 (5-28)	0.59[1]
Median length of stay (d)	10 (7-19)	9 (7-13.5)	0.22[1]
Disease extent, *n* (%)			0.072
Left-sided colitis	8 (17.8)	54 (29.8)	
Pancolitis	37 (82.2)	127 (70.2)	
Toxic megacolon, *n* (%)	0	4 (2.2)	0.41
Extraintestinal manifestations, *n* (%)	2 (4.4)	31 (17.1)	0.02
Superimposed clostridium difficile, *n* (%)	3 (6.7)	6 (3.3)	0.26
Smoking status, *n* (%)			0.037
Never	21 (46.7)	121 (66.9)	
Current	6 (13.3)	17 (9.4)	
Former	18 (40.0)	43 (23.8)	
5-aminosalicyclate use, *n* (%)			0.86
Current	24 (53.3)	100 (55.2)	
Never	14 (31.1)	49 (27.1)	
Intolerant/ceased	7 (15.6)	32 (17.7)	
Current thiopurine use, *n* (%)	6 (13.3)	29 (16.0)	0.29
Current methotrexate use, *n* (%)	1 (2.2)	2 (1.1)	0.16
Anti-TNF antagonist use, *n* (%)			0.74
Current	6 (13.3)	23 (12.7)	
Never	37 (82.2)	134 (74.0)	
Intolerant	1 (2.2)	6 (3.3)	
Secondary loss of response	1 (2.2)	18 (9.9)	
Vedolizumab use, *n* (%)			0.024
Current	8 (17.8)	9 (5.0)	
Never	35 (77.8)	163 (90.0)	
Intolerant	0	4 (2.2)	
Secondary loss of response	2 (4.4)	5 (2.8)	
Biologics on admission, *n* (%)	14 (31.0)	35 (19.4)	0.11
Oral steroids at admission, *n* (%)	15 (33.3)	78 (43.1)	0.31
Median admission UCEIS	5.5 (5-7)	6 (5-7)	0.82[1]
Median serum albumin on day of admission (g/L)	31 (27-34)	33 (29-38)	0.005[1]
Median haemoglobin on day of admission (g/L)	126 (111-135)	124 (108-139)	0.92[1]
Median platelet count on day of admission (units)	333.5 (277-386)	393 (293-500)	0.006[1]
Median CRP on day of admission (mg/L)	69 (33-121)	54 (30-99)	0.34[1]
Median admission faeces calprotectin (mcg/g)	2400 (1600-4600)	2850 (1400-5300)	0.48[1]

Median stool frequency on day of admission	10 (7-15)	10 (8-18)	0.41[1]

[1]Wilcoxon Ranksum test.

Continuous variables reported as median with interquartile range. UC: Ulcerative colitis; ASUC: Acute severe ulcerative colitis; UCEIS: Ulcerative colitis endoscopic index of severity; CRP: C-reactive protein; TNF: Tumour necrosis factor.

Table 2 Summary of primary and secondary outcomes of older *vs* younger adults with acute severe ulcerative colitis, *n* (%)

	≥ 60 yr, *n* = 45	< 60 yr, *n* = 181	Crude RD (95%CI)	Crude RR (95%CI)	Adjusted RR[1] (95%CI)	OR (95%CI)
Primary outcome: Steroid non-response	19 (42.2%)	85 (47%)	-0.47 (-0.21 to 0.11)	0.89 (0.61-1.30)	0.99 (0.34-2.90)	0.82 (0.43-1.58)
Response to medical rescue therapy	13 (76.5%)	66 (85.7%)	-0.09 (-0.31 to 0.12)	0.89 (0.67-1.17)	-	0.54 (0.16-1.85)
Colectomy same admission	6 (13.3%)	19 (10.5%)	0.028 (-0.08 to 0.13)	1.27 (0.53-2.99)	1.43 (0.34-6.06)	1.31 (0.50-3.41)
Colectomy at 3 mo	9 (20.9%)	30 (17.6%)	-0.03 (-0.10 to 0.16)	1.18 (0.61-2.3)	1.31 (0.32-5.30)	1.23 (0.54-2.80)
Colectomy at 12 mo	9 (24.3%)	42 (28.8%)	-0.04 (-0.20 to 0.11)	0.85 (0.45-1.57)	1.2 (0.29-4.97)	0.79 (0.35-1.80)

[1]Adjusted relative risk for sex, disease duration, smoking status, disease extent, current biologic use.

RD: Risk difference; RR: Relative risk; aRR: Adjusted relative risk; OR: Odds ratio.

years, a similar proportion of episodes underwent colectomy during the index admission [2/25 (8.7%) in ≥ 70 years *vs* 21/201 (8.7%) in < 70 years, P = 0.52; crude RD = -0.026 (-0.15 to 0.09); crude RR = 0.77 (0.19-3.04); adjusted RR = 0.91 (0.16-5.09), P = 0.52; crude OR = 0.74 (0-3.0), P = 0.52].

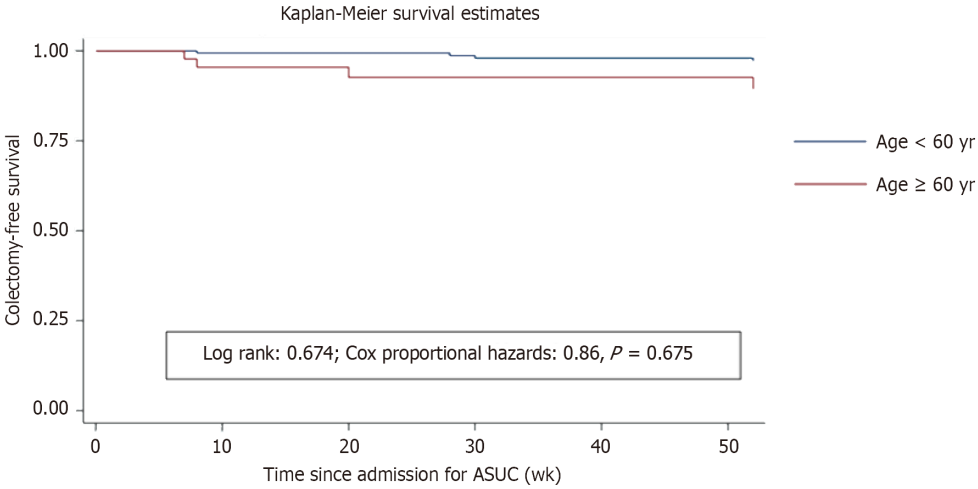
Colectomy at 3 mo: At 3 mo, 9 (20%) patients ≥ 60 years of age had undergone a colectomy, compared to 30 (17.6%) patients < 60 years of age [crude RD = -0.03 (-0.10 to 0.16); crude RR = 1.18 (0.61-2.3), P = 0.38; adjusted RR = 1.31 (0.32-0.53), P = 0.82; crude OR = 1.23 (0.54-2.80); crude HR = 1.21 (0.55-2.648, P = 0.620]. In older adults, of the 13 episodes which responded to medical rescue therapy, 1 patient with two episodes of ASUC within a three-month period of the index admission underwent a colectomy. When age cut-off was defined as 70 years, a lower proportion of episodes underwent colectomy at 3 mo [2/23 (8.7%) in ≥ 70 years *vs* 37/190 (19.5%) in < 70 years of age, P = 0.264; crude RD = -0.1.09 (-0.235 to 0.02); crude RR = 0.44 (0.11-1.73); adjusted RR = 0.72 (0.14-3.73), crude OR = 0.39 (0-1.58), P = 0.165].

Colectomy at 12 mo: At 12 mo, 9 (24.3%) patients ≥ 60 years of age had undergone a colectomy, compared to 42 (28.8%) patients < 60 years of age [crude RD = -0.04 (-0.20 to 0.11); crude RR = 0.85 (0.45-1.57), P = 0.376; adjusted RR = 1.21 (0.29-4.97), P = 0.88; crude OR = 0.79 (0.35-1.80); crude HR = 0.86 (0.43-1.71), P = 0.69]. The Kaplan-Meier curve for colectomy-free survival is shown in Figure 2. When age cut-off was defined as 70 years, a lower proportion of episodes underwent colectomy at 12 mo [2/23 (8.7%) in ≥ 70 years *vs* 49/203 (24.1%) in < 70 years of age, P = 0.042; crude RD = -0.21 (-0.35 to -0.07); crude RR = 0.29 (0.07-1.14), P = 0.026; adjusted RR = 0.63 (0.11-3.41), P = 0.673; OR = 0.23 (0-0.92)].

DISCUSSION

Although the management of IBD in older adults remains a challenge, the basic treatment paradigms across all age groups are the same. This study is one of the largest studies describing outcomes of ASUC in older adults. It demonstrates that the rates of steroid non-response as well as short and long-term colectomy risk in older adults is comparable to those who are less than 60 years of age.

There is an increasing number of older adults with IBD, correlating with both the rising incidence of IBD and the ageing population[25]. The widely accepted definition of elderly-onset IBD is disease onset at age 60 years or older[25]. Hence, this study used 60 years as the cut-off age to define older adults. In this study, 20% of patients were over 60 years of age at the time of their ASUC presentation; 15% of patients (33 out of 226) had their initial diagnosis of UC after the age of 60. This is comparable to current data showing 10%-25% of IBD patients are diagnosed after the age of 60[25,26]. Previous studies have exhibited that older adults with UC are more likely to present with a severe initial episode, display proctocolitis or limited left-sided colitis, and develop toxic megacolon which is associated with high



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Figure 2 Kaplan-Meier curve, colectomy-free survival. ASUC: Acute severe ulcerative colitis.

mortality[27,28]. In this study, 13 (28.9%) episodes had proctocolitis or limited left-sided colitis, and there were no episodes of toxic megacolon in older adults.

Traditionally, the Oxford index has been utilised to define steroid failure in patients with ASUC, and in this study the same definitions were applied[22]. Previous studies have shown that about 40% of patients with ASUC fail initial therapy with IVCS[29]. This study reconfirms that the rate of steroid failure is similar between older adults (42.2%) and the younger cohort of patients (47%). This is in contrast to a recently published multicentre Japanese study[30]. IVCS continue to be the first-line treatment option for older adults, although steroid-specific adverse effects are to be taken into consideration. Nevertheless, older adults with ASUC should not be undertreated, as poorly controlled disease and repeated courses of steroids induce undesirable outcomes. In this study, more than 75% of older adults responded to medical rescue therapy and avoided colectomy during admission for ASUC. The effectiveness of medical rescue therapy demonstrated in the current study is comparable to that demonstrated in larger randomised-controlled trials[31,32]. Of the episodes who responded to medical rescue therapy, only 1 patient had undergone a colectomy by 12 mo. Biologic agents in older adults with IBD were recently shown to have similar drug sustainability, effectiveness, and safety[33]. Older adults on IFX also have a similar risk of developing adverse effects and loss of response as younger patients [34]. Thus, medical rescue therapy can be offered judiciously to older adults.

This study has several strengths, foremost that it is one of the largest studies describing outcomes of ASUC in older adults. Although this was not a controlled trial, this cohort of patients was managed through two tertiary IBD subspeciality units which have defined treatment protocols for hospitalised ASUC patients consistent with international guidelines. Results are therefore generalisable to similar real-world clinical settings. The study has a few limitations. Firstly, the study is retrospective. Secondly, long-term safety of IFX and CsA were not studied systematically. The assessment of clinical response after initiation of rescue therapy with the Lichtiger score or Mayo score may have been beneficial. Finally, clinical and biochemical data at 12 mo may have also proved valuable for the analysis of the study.

CONCLUSION

Management of older adults with ASUC remains challenging. This study demonstrates that the rate of IVCS non-response in older adults with ASUC is similar to younger patients, and medical rescue therapy is equally effective. Clinical decisions for older adults with ASUC should still be determined by disease severity rather than chronological age alone.

ARTICLE HIGHLIGHTS

Research background

The management of older adults with acute severe ulcerative colitis (ASUC) is uniquely challenging due to their numerous medical and social factors. Up to 20% of patients with ulcerative colitis have late-onset disease with their first flare occurring after the age of 60.

Research motivation

There is minimal data available on the outcomes of older adults with ASUC. Previous studies report higher treatment failure rates in older adults who are commenced on their first biologic. We planned this study to define both short and long term outcomes for this cohort and determine if they have similar outcomes compared to the younger cohort.

Research objectives

We aimed to determine the steroid non-response rates for older adults with ASUC during index admission. We also aimed to study their response to medical rescue therapy and colectomy rates up to 12 mo from initial presentation.

Research methods

We conducted a retrospective cohort study investigating the short and long term outcomes among 226 ASUC episodes between January 2013 and July 2020 at two tertiary hospitals in Queensland, Australia. Clinical characteristics, laboratory parameters, and disease outcomes, including mortality, were compared between older and younger adults. A modified Poisson regression model was used for analysis.

Research results

The prevalence of older adults with ASUC was 19.9%. Steroid non-response rate in older adults were comparable to younger adults (42.2% *vs* 47%, $P = 0.62$). Response rates to medical rescue therapy was also comparable between the two groups (76.5% *vs* 85.7%, $P = 0.46$). Index admission colectomy (13.3% *vs* 10.5%, $P = 0.60$), colectomy at 3 mo (20% *vs* 16.6%, $P = 0.66$), and colectomy at 12 mo (20% *vs* 23.2%, $P = 0.68$) were also similar between the two groups.

Research conclusions

Older adults with ASUC have similar outcomes compared to younger patients less than 60 years of age for rates of steroid non-response, medical rescue therapy, and colectomy at index admission, 3 and 12 mo.

Research perspectives

Clinical decisions for older adults with ASUC remains to be a challenge however should still be determined by disease severity rather than chronological age alone. Future prospective studies will allow further improvement in their management.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Liver histopathological lesions is severe in patients with normal alanine transaminase and low to moderate hepatitis B virus DNA replication

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Abstract

BACKGROUND

Chronic hepatitis B virus (HBV) infection remains a major global public health problem. Chronic hepatitis B (CHB) patients can be divided into treatment indication and non-treatment indication individuals according to alanine transaminase (ALT), HBV DNA, serum hepatitis B e antigen status, disease status [liver cirrhosis, hepatocellular carcinoma (HCC), or liver failure], liver necroinflammation or fibrosis, patients' age, and family history of HCC or cirrhosis. For example, normal ALT patients in 'immune-tolerant' phase with HBV DNA higher than 10^7 or 2×10^7 IU/mL, and those in 'inactive-carrier' phase with HBV DNA lower than 2×10^3 IU/mL do not require antiviral therapy. However, is it reasonable to set the defined values of HBV DNA as the fundamental basis to estimate the disease state and to determine whether to start treatment? In fact, we should pay more attention to those who do not match the treatment indications (gray-zone patients both in the indeterminate phase and in the 'inactive-carrier' phase).

AIM

To analyze the correlation of HBV DNA level and liver histopathological severity, and to explore the significance of HBV DNA for CHB with normal ALT.

METHODS

From January 2017 to December 2021, a retrospective cross-sectional set of 1299 patients with chronic HBV infection (HBV DNA > 30 IU/mL) who underwent liver biopsy from four hospitals, including 634 with ALT less than 40 U/L. None of the patients had received anti-HBV treatment. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system. On the basis of the HBV DNA level, patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL [7.00 Log IU/mL, the European Association for the Study of the Liver (EASL) guidelines] or ≤ 2 × 10^7 IU/mL [7.30 Log IU/mL, the Chinese Medical Association (CMA) guidelines]; high replication group, HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL. Relevant factors (demographic characteristics, laboratory parameters and noninvasive models) for liver histopathological severity were analyzed by univariate analysis, logistics analysis and propensity score-matched analysis.

RESULTS

At entry, there were 21.45%, 24.29%, and 30.28% of the patients had liver histopathological severities with ≥ A2, ≥ F2, and ≥ A2 or/and ≥ F2, respectively. HBV DNA level (negative correlation) and noninvasive model liver fibrosis 5 value (positive correlation) were independent risk factors for liver histopathological severities (liver necroinflammation, liver fibrosis, and treatment indication). The AUROCs of the prediction probabilities (PRE_) of the models mentioned above (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) were 0.814 (95%CI: 0.770-0.859), 0.824 (95%CI: 0.785-0.863), and 0.799 (95%CI: 0.760-0.838), respectively. HBV DNA level (negative correlation) was still an independent risk factor when diagnostic models were excluded, the *P* values (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) were 0.011, 0.000, and 0.000, respectively. For the propensity score-matched pairs, whether based on EASL guidelines or CMA guidelines, the group with significant liver histology damage (≥ A2 or/and ≥ F2) showed much lower HBV DNA level than the group with non- significant liver histology damage (< A2 and < F2). Patients in the moderate replication group (with indeterminate phase) had the most serious liver disease pathologically and hematologically, followed by patients in the low replication group (with 'inactive-carrier' phase) and then the high replication group (with 'immune-tolerant' phase).

CONCLUSION

HBV DNA level is a negative risk factor for liver disease progression. The phase definition of CHB may be revised by whether the level of HBV DNA exceeds the detection low limit value. Patients who are in the indeterminate phase or 'inactive carriers' should receive antiviral therapy.

Key Words: Chronic hepatitis B; Hepatitis B virus DNA; Histology; Risk factors

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Core Tip: According to the guidelines, for patients with normal alanine transaminase (ALT), hepatitis B virus (HBV) DNA levels were defined as ≥ 10^7/2 × 10^7 and < 2 × 10^3 IU/mL in the 'immune-tolerant' and the 'inactive-carrier' phase, respectively. However, it is still controversial. In this study, we analyzed the liver histopathology and the risk factors in 634 cases with positive HBV DNA and normal ALT. We found that patients with low or moderate HBV DNA level had more severe liver diseases. HBV DNA level (negative correlation) was an independent risk factor for liver histopathological severity. Therefore, we consider that the phase definition of chronic hepatitis B may be revised based on whether the level of HBV DNA exceeds the detection low limit value.

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INTRODUCTION

With the promotion of the hepatitis B vaccine or combined hepatitis B immune globulin, new hepatitis B virus (HBV) infections are decreasing. However, there are still about 257 million people infected with

HBV worldwide[1], and about 887000 people died from HBV infection each year, of which liver cirrhosis and hepatocellular carcinoma (HCC) deaths account for 52% and 38%, respectively[2]. It's undeniable that chronic HBV infection is still a major global public health problem. Therefore, the World Health Organization (WHO) has proposed the global health sector strategy of 'eliminating viral hepatitis as a major public health threat by 2030'. One of the goals is to achieve a diagnosis rate to 90% and a treatment rate to 80% of HBV infection by 2030[3]. China had made great progress in reducing HBV infections, but the challenges still remain. Currently, there are still 70 million chronic HBV infections in China[2].

The natural history of chronic HBV infection can be generally divided into four phases: Hepatitis B e antigen (HBeAg) positive chronic HBV infection/'immune-tolerant' phase, HBeAg positive chronic hepatitis B (CHB)/immune-clearance phase, HBeAg negative chronic HBV infection/'inactive-carrier' phase, and HBeAg negative CHB/reactivation phase[2,4,5].

The disease progression and treatment indications judgement is mainly based on serum HBeAg status, HBV DNA level, alanine transaminase (ALT) level, and severity of liver disease, combined with patients' age, family history, and accompanying diseases[2,4-7]. In accordance with the European Association for the Study of the Liver (EASL) guidelines[4] and the CHB treatment algorithm in the United States[5], regardless of HBeAg status, patients with HBV DNA > 2×10^3 IU/mL, ALT > upper limit of normal (ULN), and/or at least moderate liver necrotic inflammation or liver fibrosis should be treated. However, if HBV DNA is less than 2×10^3 IU/mL, how to deal with it clinically becomes an issue. According to the EASL guidelines[4] and the CHB treatment algorithm in the United States[5], CHB patients with normal ALT in the immune tolerant phase refer to HBV DNA > 10^7 IU/mL, in the 'inactive-carrier' phase refer to HBV DNA < 2×10^3, and in the indeterminate phase refer to $2 \times 10^3 \leq$ HBV DNA $\leq 10^7$ IU/mL. According to the Chinese Medical Association (CMA) guidelines[2], patients in the immune tolerant phase refer to HBV DNA > 2×10^7 IU/mL, in the 'inactive-carrier' phase refer to HBV DNA < 2×10^3, and in the indeterminate phase refer to $2 \times 10^3 \leq$ HBV DNA $\leq 2 \times 10^7$ IU/mL.

Although HBV DNA is an important indicator for judging disease progression and treatment indications, the reported conclusions about HBV DNA and disease severity remain controversial[8-14]. Moreover, the 'gray-zone' and/or the indeterminate phase population should not be ignored with the consideration of the guidelines. The aim of this study was to find the correlation of clinical and laboratory parameters with liver histopathological severity in 634 CHB patients with ALT < ULN who required liver biopsy to assess liver inflammation and fibrosis. Studies on liver pathological changes in the 'gray-zone' and/or the indeterminate phase population and the identification of the risk factors for disease progression might be of great significance.

MATERIALS AND METHODS

Data collection

From January 2017 to December 2021, there were 1299 chronic HBV infections (including 634 with ALT < ULN) who underwent liver biopsy were included in this retrospective cross-sectional study conducted in four hospitals. The patients were hospitalized in the Department of Hepatology, Ningbo No. 2 Hospital; the Department of Infectious Diseases, Xiangshan Hospital Affiliated to Wenzhou Medical University; the Department of Infectious Diseases, The First Hospital of Ninghai County; and the Department of Infectious Diseases, the Affiliated Yangming Hospital of Ningbo University, Ningbo, China.

The inclusion criteria were as follows: Patients aged 13-78 years, HBsAg positivity for at least 6 mo, HBV DNA ≥ 30 IU/mL, and no previous anti-HBV treatment. The ULN of ALT was 40 U/L according to the WHO/EASL/Asian Pacific Association for the Study of the Liver guidelines[4,6,7]. The exclusion criteria were as follows: Co-infection with hepatitis C virus, hepatitis D virus, hepatitis E virus, and human immunodeficiency virus; autoimmune hepatitis; Wilson's disease; nonalcoholic fatty liver disease; chronic alcohol consumption (> 30 g/d for men and > 20 g/d for women[15]); and incomplete data.

This study was approved by the ethics committee of Ningbo No. 2 Hospital (PJ-NBEY-KY-2017-069-01, PJ-NBEY-KY-2021-037-02, and PJ-NBEY-KY-2022-138-01). In this study, medical data was obtained from previous clinical diagnosis and treatment, and informed consent was exempted.

The clinical data was collected within one week before liver biopsy. Demographic characteristics and laboratory data, including age, sex, albumin (ALB), globulin (GLB), ALB–GLB ratio (AGR), ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), white blood cell (WBC), neutrophil–lymphocyte ratio (NLR), platelet (PLT), HBeAg, HBV DNA, and noninvasive models such as aspartate transaminase to platelet ratio index (APRI)[16], fibrosis-4 (FIB-4)[17], liver inflammation and fibrosis-5 (LIF-5)[18], were recorded.

Blood test

Blood routine was detected using Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Japan). Serum liver function was detected with Simens Advia Chemistry XPT system

analyzer (Siemens Healthcare, Germany). Serum HBV DNA was measured by real-time fluorescence quantitative PCR (ABI7500, Applied Biosystems, CA, USA) and HBV nucleic acid quantitative detection kit (DAAN Gene Co., Ltd. Sun Yat-sen University, China) with the lowest detection value of 30 IU/mL. According to the HBV DNA level, patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL (7.00 Log IU/mL) or ≤ 2 × 10^7 IU/mL (7.30 Log IU/mL); and high replication group, HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL[2,4,5]. HBsAg and HBeAg were detected by chemiluminescence method (Abbott AxSYM System, IL, United States). In this study, HBeAg was presented as 1 for positive and 0 for negative. The same quality control standards were employed.

Liver histological examination

The biopsy device (BARD Magnum, United States) comprised a biopsy gun (with the tissue length of 22 mm) and a biopsy needle (18G). All patients had no liver biopsy contraindications and signed informed consent forms. Liver biopsy was performed under the guidance of color Doppler ultrasound. Liver tissue samples of more than 2 cm in length and more than 6 intact portal veins were required. The liver specimens were first assessed by two pathology experts from the hospital and then by a senior pathologist from the Department of Pathology, Fudan University, China. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system[19]. A Metavir necroinflammatory activity score of ≥ 2 (A2) and ≥ 3 (A3) indicated significant and severe liver inflammation, respectively. A Metavir fibrosis score of ≥ 2 (F2), ≥ 3 (F3), and ≥ 4 (F4) indicated significant liver fibrosis, advanced fibrosis, and cirrhosis, respectively. In accordance with the guidelines, the treatment indications of patients with ALT < ULN were ≥ A2 or/and ≥ F2. Hence, these patients were divided into treatment indication group and nontreatment indication group (< A2 and < F2).

Statistical analysis

Propensity score-matched analysis was used to reduce the effect of selection bias and potential confounding between the two groups. According to the HBV DNA levels of EASL and CMA guidelines, the low/moderate replication group and high replication group were matched at a ratio of 1:1 (nearest neighbor matching within caliper) based on sex, age, ALB, GLB, AGR, ALT, AST, ALP, GGT, WBC, NLR, PLT, APRI, FIB-4, and LIF-5.

The data were analyzed *via* SPSS software version 22.0 (SPSS Inc., IL, United States). HBV DNA levels were expressed as logarithms. The normally distributed variables were presented as means with standard deviations analyzed by using independent-samples *t* test (two datasets). The non-normal distribution variables were expressed as medians (Q1-Q3) analyzed by using nonparametric tests (Mann-Whitney *U* test) for two datasets. The chi-square test was used for categorical data. Ridit analysis and Spearman's rank correlation analysis were used for ranked data. The binary logistic regression analysis was performed taking liver histopathological severity (A and F) as the dependent variables and relevant factors (*P* value < 0.1) as independent variables. The dependent variables were < A2 *vs* ≥ A2, < F2 *vs* ≥ F2, and nontreatment indication *vs* treatment indication. The relevant factors were analyzed, and the diagnostic value was evaluated using receiver operating characteristic (ROC) curve and the area under the ROC curve (AUROC). All tests were two tailed, and statistical significance was set at *P* value < 0.05.

RESULTS

Enrolled patients

There were 50 patients were excluded due to incomplete data of liver pathology, HBeAg, ALB, ALP, WBC, neutrophils, and lymphocytes, and 615 patients were excluded as ALT > 1 × ULN. The flow diagram of the study population is shown in Figure 1. Finally, 634 patients were included in the study, among which 336 (EASL guidelines) and 377 (CMA guidelines) were classified into the low/moderate replication group, including 49 Low-replication (HBV DNA < 2 × 10^3); 298 (EASL guidelines) and 257 (CMA guidelines) were divided into the high replication group.

Baseline characteristics

The baseline characteristics of 634 patients were divided according to liver pathology which are shown in Table 1. The mean age of the participants was 35.61 ± 10.30 years, the mean ALT, AST, and HBV DNA levels were 23.77 ± 8.58 U/L, 24.15 ± 8.91 U/L, and 6.18 ± 1.87 Log IU/mL, respectively. Among these patients, 349 (55.05%) were men and 432 (68.14%) were HBeAg positive.

Patients with liver inflammation A0, A1, A2, and A3 were 117 (18.45%), 381 (60.10%), 97 (15.30%), and 39 (6.15%), with liver fibrosis F0, F1, F2, F3, and F4 were 148 (23.34%), 332 (52.37%), 87 (13.72%), 37 (5.84%), and 30 (4.73%), respectively. Patients with ≥ A2 accounted for 21.45% (136 patients), ≥F2 for 24.29% (154 patients), and treatment indication (≥ A2 or/and ≥ F2) for 30.28% (192 patients).

Table 1 Distribution of baseline characteristics in 634 chronic hepatitis B patients with alanine transaminase < upper limit of normal

Parameters	All patients (*n* = 634)	HBV DNA levels (EASL guidelines)				HBV DNA levels (CMA guidelines)			
		Low/moderate replication (*n* = 336)	High replication (*n* = 298)	$\chi^2/t/Z/u$	*P* value	Low/moderate replication (*n* = 377)	High replication (*n* = 257)	$\chi^2/t/Z/u$	*P* value
Age, mean ± SD, yr	35.61 ± 10.30	38.09 ± 9.99	32.82 ± 9.95	6.640	< 0.001	37.34 ± 10.17	33.07 ± 9.99	5.222	< 0.001
Male, *n* (%)	349 (55.05)	192 (57.14)	157 (52.68)	1.269	0.260	209 (55.44)	140 (54.47)	0.057	0.811
HBeAg positive, *n* (%)	432 (68.14)	142 (42.26)	290 (97.31)	220.486	< 0.001	180 (47.74)	252 (98.05)	178.165	< 0.001
ALB, mean ± SD, g/L	42.72 ± 4.45	42.91 ± 4.97	42.49 ± 3.78	1.189	0.235	42.75 ± 4.88	42.67 ± 3.74	0.220	0.826
GLB, mean ± SD, g/L	27.82 ± 4.20	27.96 ± 4.11	27.66 ± 4.30	0.893	0.372	28.11 ± 4.19	27.40 ± 4.18	2.105	0.036
AGR, mean ± SD	1.57 ± 0.29	1.57 ± 0.30	1.57 ± 0.28	-0.148	0.882	1.56 ± 0.30	1.59 ± 0.28	-1.581	0.114
ALT, mean ± SD, U/L	23.77 ± 8.58	24.84 ± 8.66	22.56 ± 8.34	3.372	0.001	24.38 ± 8.69	22.87 ± 8.35	2.186	0.029
AST, mean ± SD, U/L	24.15 ± 8.91	25.00 ± 7.52	23.20 ± 10.18	2.558	0.011	25.04 ± 10.28	22.85 ± 6.17	3.069	0.002
ALP, mean ± SD, U/L	71.26 ± 26.06	71.52 ± 24.85	70.96 ± 27.39	0.269	0.788	71.21 ± 24.99	71.32 ± 27.60	-0.054	0.957
GGT, median (Q1-Q3), U/L	18.00 (13.00-25.00)	20.00 (15.00-30.00)	16.00 (13.00-23.00)	4.966	< 0.001	19.00 (14.00-29.00)	16.00 (13.00-23.00)	4.069	< 0.001
WBC count, mean ± SD, $\times 10^9$/L	5.39 ± 1.42	5.33 ± 1.39	5.47 ± 1.45	-1.208	0.228	5.33 ± 1.44	5.48 ± 1.39	-1.321	0.187
NLR, mean ± SD	2.03 ± 1.24	2.05 ± 1.37	2.01 ± 1.08	0.443	0.658	2.04 ± 1.33	2.01 ± 1.09	0.298	0.766
PLT count, mean ± SD, $\times 10^9$/L	175.67 ± 48.83	163.93 ± 47.39	188.90 ± 47.09	-6.641	< 0.001	166.86 ± 47.55	188.58 ± 47.90	-5.629	< 0.001
HBV DNA, mean ± SD, log IU/mL	6.18 ± 1.87	4.68 ± 1.26	7.88 ± 0.50	-41.111	< 0.001	4.95 ± 1.41	7.99 ± 0.44	-33.427	< 0.001
APRI, median (Q1–Q3)	0.33 (0.25-0.44)	0.36 (0.28-0.51)	0.29 (0.23-0.38)	6.936	< 0.001	0.36 (0.27-0.48)	0.30 (0.23-0.38)	5.725	< 0.001
FIB-4, median (Q1-Q3)	0.99 (0.69-1.38)	1.13 (0.83-1.61)	0.80 (0.58-1.13)	8.109	< 0.001	1.09 (0.79-1.56)	0.81 (0.59-1.13)	6.877	< 0.001
LIF-5, mean ± SD	0.40 ± 0.15	0.45 ± 0.15	0.36 ± 0.14	7.832	< 0.001	0.44 ± 0.15	0.35 ± 0.14	7.195	< 0.001
Liver inflammatory activity									
A0, *n* (%)	117 (18.45)	58 (17.26)	59 (19.80)	4.189	< 0.001	61 (16.18)	56 (21.79)	4.426	< 0.001
A1, *n* (%)	381 (60.10)	174 (51.79)	207 (69.46)			206 (54.64)	175 (68.09)		
A2, *n* (%)	97 (15.30)	73 (21.73)	24 (8.05)			78 (20.69)	19 (7.39)		
A3, *n* (%)	39 (6.15)	31 (9.23)	8 (2.68)			32 (8.49)	7 (2.72)		
≥ A2, *n* (%)	136 (21.45)	104 (30.95)	32 (10.74)	38.299	< 0.001	110 (29.18)	26 (10.12)	32.952	< 0.001
Liver fibrosis									
F0, *n* (%)	148 (23.34)	61 (18.15)	87 (29.19)	6.382	< 0.001	70 (18.57)	78 (30.35)	6.053	< 0.001
F1, *n* (%)	332 (52.37)	153 (45.54)	179 (60.07)			179 (47.48)	153 (59.53)		
F2, *n* (%)	87 (13.72)	65 (19.35)	22 (7.38)			70 (18.57)	17 (6.61)		
F3, *n* (%)	37 (5.84)	29 (8.63)	8 (2.68)			30 (7.96)	7 (2.72)		

F4, *n* (%)	30 (4.73)	28 (8.33)	2 (0.67)			28 (7.43)	2 (0.78)		
≥ F2, *n* (%)	154 (24.29)	122 (36.31)	32 (10.74)	56.155	< 0.001	128 (33.95)	26 (10.12)	47.212	< 0.001
Treatment indication									
< A2 and < F2, *n* (%)	442 (69.72)	191 (56.85)	251 (84.23)	56.089	< 0.001	224 (59.42)	218 (84.82)	46.730	< 0.001
≥ A2 or/and ≥ F2, *n* (%)	192 (30.28)	145 (43.15)	47 (15.77)			153 (40.58)	39 (15.18)		

Quantitative data of normal distribution were expressed as mean ± standard deviation, non-normal distribution data were expressed as median (Q1-Q3), and categorical data were expressed as frequency and percentage. A: Liver inflammatory activity; AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver; F: Liver fibrosis; GLB: Globulin; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell.

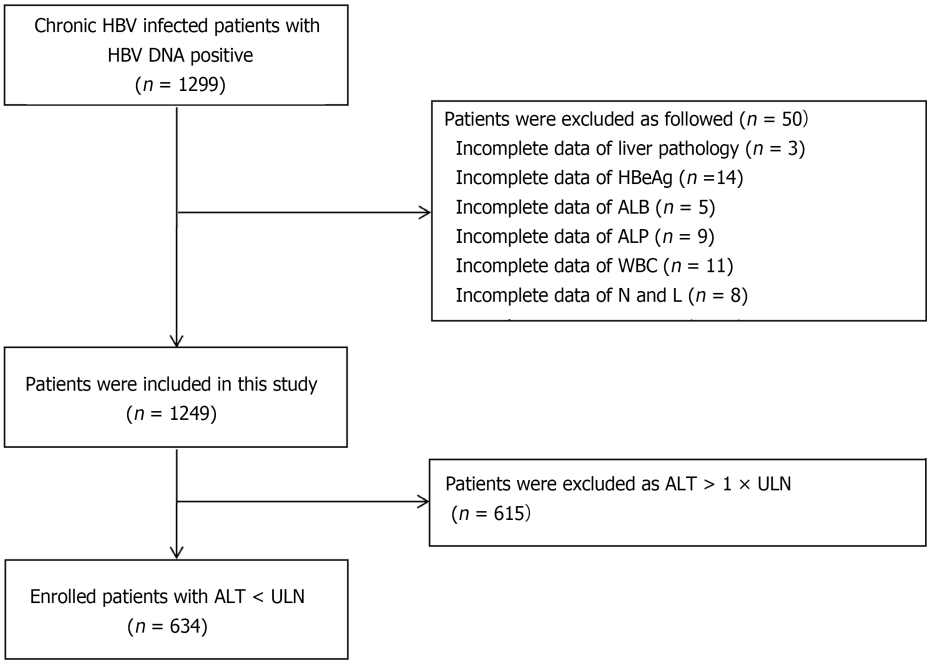


Figure 1 Flow diagram of enrolled patients. ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; L: Lymphocyte; N: Neutrophils; WBC: White blood cell; HBV: Hepatitis B virus; ULN: Upper limit of normal.

Comparisons of clinical indexes and liver pathological changes among 634 patients with different HBV DNA levels (EASL and CMA guidelines)

According to the EASL and CMA guidelines, the low/moderate replication group was comprised of 336 and 298 patients while the high replication group comprised 377 and 257 patients. Patients with high HBV DNA levels had a higher HBeAg-positive composition for both the two guidelines (χ^2 = 220.486 and 178.165, P < 0.001). Compared with the low/moderate replication group, patients in the high replication group had lower age, ALT, AST, GGT, APRI, FIB-4, and LIF-5, and higher PLT. The results were detailed in Table 1.

In general, liver histopathological severity degree in the low/moderate replication group was of the most serious forms regardless of liver inflammation or liver fibrosis. The average Ridit values of necroinflammatory activity grading in the low/moderate replication group, and high replication group were 0.544 and 0.451 (EASL guidelines), 0.541 and 0.440 (CMA guidelines), respectively, with statistically significant differences (u = 4.189, 4.426; r = -0.183, -0.194; P < 0.001). The average Ridit values of liver fibrosis staging in the low/moderate replication group and high replication group were 0.567 and 0.424 (EASL guidelines), 0.556 and 0.418 (CMA guidelines), respectively, with statistically significant differences (u = 6.382, 6.053; r = -0.271, -0.257; P < 0.001). There were 104 (30.95%) and 32 (10.74%) (EASL guidelines), 110 (29.18%) and 26 (10.12%) (CMA guidelines) patients with liver inflammatory activity ≥ 2 (≥ A2) in the two groups, respectively (χ^2 = 38.299, 32.952; P < 0.001). Patients with liver fibrosis ≥ 2 (≥ F2) in the two groups were 122 (36.31%) and 32 (10.74%) (EASL guidelines), 128 (33.95%) and 26

(10.12%) (CMA guidelines), respectively (χ^2 = 56.155, 47.212; P < 0.001). Simultaneously, the number of patients with treatment indication (≥ A2 or/and ≥ F2) in the two groups were 145 (43.15%) and 47 (15.77%) by the EASL guidelines, or 153 (40.58%) and 39 (15.18%) by the CMA guidelines (χ^2 = 56.089, 46.730; P < 0.001). The results are displayed in Table 1 and Figure 2.

In addition, there were 16 (32.65%), 20 (40.82%), and 22 (44.90%) patients with liver inflammatory activity ≥ 2 (≥ A2), liver fibrosis ≥ 2 (≥ F2), and treatment indication (≥ A2 or/and ≥ F2) in the low-replication (HBV DNA < 2 × 10^3, 49 cases), respectively.

Predictors of significant liver histology in 634 patients

The univariate analysis indicated that the statistically significant variables which could affect liver inflammation activity, liver fibrosis, and treatment indications were age, HBeAg, ALB, GLB, AGR, ALT, AST, GGT, PLT, HBV DNA, APRI, FIB-4, and LIF-5. The results are demonstrated in Table 2.

The logistics analysis showed that HBV DNA level (negative correlation), age (negative correlation), GGT level (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for liver inflammation activity; HBV DNA level (negative correlation), GGT level (positive correlation), APRI value (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for liver fibrosis; HBV DNA level (negative correlation), APRI value (positive correlation), and LIF-5 value (positive correlation) were independent risk factors for treatment indications. The AUROC of the prediction probabilities (PRE_) of the abovementioned models (< A2 *vs* ≥ A2, < F2 *vs* ≥ F2, < A2 and < F2 *vs* ≥ A2 or/and ≥ F2) was 0.814 (95%CI: 0.770-0.859), 0.824 (95%CI: 0.785-0.863), and 0.799 (95%CI: 0.760-0.838), respectively. Considering that the diagnostic models such as APRI, FIB-4, and LIF-5 contained some of the indices, the HBV DNA level (negative correlation) was still an independent risk factor for the dependent variables after the diagnostic models were eliminated. The results are listed in Table 3 and Figure 3.

Predictors of significant liver histology in propensity score-matched pairs

To minimize the effect of potential confounders in the comparison of liver histology damages (< A2 and < F2 *vs* ≥ A2 or/and ≥ F2) between the low/moderate replication group and high replication group, we matched 316 pairs (EASL guidelines) and 277 pairs (CMA guidelines) of patients by propensity score-matching. In these pairs, there were no significant differences between the low/moderate replication and high replication groups (P > 0.05) in the baseline characteristics (sex, age, ALB, GLB, AGR, ALT, AST, ALP, GGT, WBC, NLR, PLT, APRI, FIB-4, and LIF-5) (Table 4), hence achieving covariate balance. For the propensity score-matched pairs, both EASL and CMA guidelines, the group of significant liver histology damage (≥ A2 or/and ≥ F2) had much lower HBV DNA levels than that of the non- significant liver histology damage group (< A2 and < F2) (EASL guidelines: 5.81 ± 1.23 Log IU/mL *vs* 7.90 ± 0.49 Log IU/mL, t = -27.967, P < 0.001; CMA guidelines: 5.78 ± 1.42 Log IU/mL *vs* 8.01 ± 0.43 Log IU/mL, t = -24.922, P < 0.001) (Table 4).

DISCUSSION

At present, chronic HBV infections are classified by treatment indications which are based mainly on serum HBV DNA, ALT, and liver disease severity[2,4-7]. The treatment indications were easy to identify clinically. However, we should pay more attention to those not meeting treatment indications (the so-called gray-zone patients), and there is a considerable number of such people. One retrospective cohort study[20] involved 3366 CHB patients came from 5 clinical centers of America and 7 towns of Taiwan, China which were followed up for at least 1 year and the mean time was 12.5 years. Staging of the disease was determined according to the American Association for the Study of Liver Diseases (AASLD) 2018 hepatitis B guidance[21]. The result showed that patients in the indeterminate phase count for 50.9% in American cohort and 31.8% in Taiwan, China with an average of 38.7%. Yao *et al*[22] also adopted the same guidelines (ALT < ULN, male for 35 U/L and female for 25 U/L), and 4759 CHB patients in Nanjing, China were included among which 27.8% were in the indeterminate phase.

According to the guidelines/CHB treatment algorithm in the United States[2,4,5], the 'gray-zone' population is defined as the following: (1) ALT < ULN and HBV DNA < 2 × 10^3 IU/mL (most are HBeAg negative, that is, inactive CHB or HBeAg-negative HBV infection); (2) ALT continues to be normal, 2 × 10^3 ≤ HBV DNA ≤ 10^7 IU/mL (EASL guidelines) or ≤ 2 × 10^7 IU/mL (CMA guidelines), that is, CHB in the indeterminate phase; and (3) immune-tolerant CHB (HBV DNA > 10^7 IU/mL or > 2 × 10^7 IU/mL).

Actually, it is an indisputable fact that a high proportion of the 'gray-zone' population still have disease progression[8,23-30]. A previous study found that in the 'gray-zone' population with ALT < 2 × ULN, 510 of 1148 patients (44.42%) had liver pathological changes ≥ A2 or/and ≥ F2, and in those with ALT < 1 × ULN, nearly 30% had liver pathological changes ≥ A2 or/and ≥ F2[31], regardless of the ULN cutoff of ALT (50 U/L or 30 U/L for men; 40 U/L or 19 U/L for women). In this study, among 634 patients with ALT < ULN, 136 (21.45%) had liver inflammation ≥ A2, 154 (24.29%) had liver fibrosis ≥ F2, and 192 (30.28%) had treatment indications (≥ A2 or/and ≥ F2). The judgment of treatment

Table 2 Comparison of parameters among patients with alanine transaminase < upper limit of normal and different pathological states (Entire cohort)

Parameters	Liver inflammatory activity		Liver fibrosis		Treatment indication		*P* value
	< A2 (*n* = 498)	≥ A2 (*n* = 136)	< F2 (*n* = 480)	≥ F2 (*n* = 154)	< A2 and < F2 (*n* = 442)	≥ A2 or/and ≥ F2 (*n* = 192)	
Age, mean ± SD, yr	34.68 ± 9.88	39.02 ± 11.10	34.36 ± 9.86	39.52 ± 10.71	34.28 ± 9.84	38.69 ± 10.70	< 0.001, < 0.001, < 0.001
Male, *n* (%)	268 (53.82)	81 (59.56)	253 (52.71)	96 (62.34)	234 (52.94)	115 (59.90)	0.232, 0.037, 0.105
HBeAg positive, *n* (%)	354 (71.08)	78 (57.35)	350 (72.92)	82 (53.25)	323 (73.08)	109 (56.77)	0.002, < 0.001, < 0.001
ALB, mean ± SD, g/L	43.06 ± 3.96	41.45 ± 5.75	42.96 ± 4.32	41.95 ± 4.75	43.06 ± 4.01	41.92 ± 5.24	< 0.001, 0.014, 0.003
GLB, mean ± SD, g/L	27.46 ± 4.09	29.14 ± 4.35	27.54 ± 4.06	28.70 ± 4.51	27.49 ± 4.08	28.57 ± 4.38	< 0.001, 0.003, 0.003
AGR, mean ± SD	1.60 ± 0.27	1.46 ± 0.33	1.59 ± 0.28	1.50 ± 0.31	1.60 ± 0.27	1.50 ± 0.32	< 0.001, 0.001, < 0.001
ALT, mean ± SD, U/L	22.81 ± 8.48	27.26 ± 8.07	22.80 ± 8.48	26.80 ± 8.20	22.45 ± 8.43	26.80 ± 8.16	< 0.001, < 0.001, < 0.001
AST, mean ± SD, U/L	22.93 ± 6.24	28.62 ± 14.26	22.79 ± 6.41	28.41 ± 13.26	22.64 ± 6.26	27.63 ± 12.45	< 0.001, < 0.001, < 0.001
ALP, mean ± SD, U/L	68.68 ± 24.45	80.67 ± 29.48	68.62 ± 23.35	79.49 ± 31.80	68.58 ± 23.86	77.41 ± 29.68	< 0.001, < 0.001, < 0.001
GGT, mean ± SD, U/L	20.24 ± 13.58	34.91 ± 33.78	19.87 ± 13.63	34.34 ± 31.87	19.91 ± 13.78	31.38 ± 29.60	< 0.001, < 0.001, < 0.001
WBC count, mean ± SD, × 10^9/L	5.40 ± 1.41	5.33 ± 1.53	5.43 ± 1.44	5.26 ± 1.40	5.41 ± 1.42	5.33 ± 1.46	0.622, 0.210, 0.491
NLR, mean ± SD	2.05 ± 1.21	1.96 ± 1.35	2.07 ± 1.31	1.91 ± 0.99	2.04 ± 1.21	2.02 ± 1.32	0.447, 0.158, 0.824
PLT count, mean ± SD, × 10^9/L	184.72 ± 44.67	142.52 ± 49.26	186.16 ± 44.28	142.94 ± 47.99	187.73 ± 43.49	147.90 ± 49.24	< 0.001, < 0.001, < 0.001
HBV DNA, mean ± SD, log IU/mL	6.35 ± 1.91	5.56 ± 1.59	6.44 ± 1.87	5.39 ± 1.64	6.47 ± 1.88	5.52 ± 1.67	< 0.001, < 0.001, < 0.001
APRI, mean ± SD	0.33 ± 0.14	0.59 ± 0.42	0.32 ± 0.13	0.58 ± 0.39	0.32 ± 0.12	0.54 ± 0.37	< 0.001, < 0.001, < 0.001
FIB-4, mean ± SD	1.01 0.53	1.81 ± 1.34	0.97 ± 0.49	1.81 ± 1.28	0.96 ± 0.49	1.67 ± 1.21	< 0.001, < 0.001, < 0.001
LIF-5, mean ± SD	0.37 ± 0.13	0.54 ± 0.17	0.36 ± 0.12	0.53 ± 0.16	0.36 ± 0.12	0.51 ± 0.16	< 0.001, < 0.001, < 0.001

Quantitative data of normal distribution were expressed as mean ± standard deviation, and categorical data were expressed as frequency and percentage. A: Liver inflammatory activity; AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; F: Fibrosis; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; GLB, globulin; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell.

indications is not only based on ALT level, although ALT is the most commonly used surrogate indicator reflecting liver cell damage. In addition, other surrogate markers including non-invasive tests have been rapidly developed[32-35]. In current, APRI and FIB-4 are the most widely used diagnostic models, but they are not that accurate in assessing the degree of HBV-related liver fibrosis[36]. In a previous study, a linear diagnosis model LIF-5 [LIF-5 = 0.725 + 0.005 × age + 0.003 × ALT + 0.004 × AST - 0.201 × (A/G) - 0.002 × PLT (10^9/L)][18] was constructed for the treatment indication judgment (A≥ 2 and/or F ≥ 2) of CHB patients with ALT < 2 × ULN, which had higher diagnostic value than APRI and FIB-4. This study also confirmed that the LIF-5 value (positive correlation) was an independent risk factor for liver inflammation activity, liver fibrosis, and treatment indication in CHB patients with ALT < ULN.

Table 3 Predictors of significant liver histology in 634 patients with chronic hepatitis B and alanine transaminase < upper limit of normal detected using a step-forward binary logistic regression model

Variables		Regression coefficient	Standard error	Wald's value	*P* value	OR	OR 95%CI	
							Upper limit	Lower limit
< A2 *vs* ≥ A2	Age	-0.039	0.014	7.853	0.005	0.962	0.936	0.988
	GGT	0.015	0.006	5.970	0.015	1.015	1.003	1.027
	HBV DNA	-0.147	0.064	5.306	0.021	0.863	0.761	0.978
	LIF-5	9.616	1.152	69.727	0.000	15002.994	1570.174	143353.477
	Constant	-3.643	0.685	28.240	0.000	0.026		
Eliminate diagnostic models	HBV DNA	-0.161	0.063	6.429	0.011	0.851	0.752	0.964
< F2 *vs* ≥ F2	GGT	0.016	0.006	5.968	0.015	1.016	1.003	1.029
	HBV DNA	-0.229	0.061	14.251	0.000	0.796	0.707	0.896
	APRI	2.747	0.921	8.890	0.003	15.593	2.563	94.859
	LIF-5	4.759	1.285	13.708	0.000	116.591	9.390	1447.723
	Constant	-3.392	0.577	34.608	0.000	0.034		
Eliminate diagnostic models	HBV DNA	-0.247	0.061	16.523	0.000	0.781	0.693	0.880
< A2 and < F2 *vs* ≥ A2 or/and ≥ F2	HBV DNA	-0.199	0.055	13.233	0.000	0.820	0.737	0.913
	APRI	3.124	0.888	12.393	0.000	22.747	3.995	129.530
	LIF-5	4.733	1.182	16.038	0.000	113.618	11.207	1151.897
	Constant	-2.903	0.522	30.931	0.000	0.055		
Eliminate diagnostic models	HBV DNA	-0.221	0.056	15.782	0.000	0.801	0.718	0.894

A: Liver inflammatory activity; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; F: Fibrosis; GGT: Gamma-glutamyl transpeptidase; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; HBV: Hepatitis B virus.

The univariate analysis suggested that the above indexes or diagnostic models, such as gender male, HBeAg negativity, increase in age, GLB, ALT, AST, GGT, APRI, FIB-4, and LIF-5, and decrease in ALB, AGR and PLT were correlated with the liver histopathological severity. However, during the logistic regression analysis, only HBV DNA (negative correlation) and LIF-5 (positive correlation) were independent risk factors for liver histopathological severity (liver inflammatory activity, liver fibrosis, and treatment indication). After excluding the diagnostic models, HBV DNA (negative correlation) was still an independent risk factor for the dependent variables mentioned above. Regardless of ALT level, both entire cohort and propensity score-matched pairs, patients in the low/moderate replication group had more serious liver disease (including liver pathological changes and hematological indicators). And patients with A ≥ 2, F ≥ 2, and treatment indication (≥ A2 or/and ≥ F2) in the low-replication group (HBV DNA < 2 × 10^3) accounted for 32.65%, 40.82%, and 44.90%, respectively, while patients in the high replication group had relatively mild pathological changes. In the entire cohort, the mean HBV DNA levels were 6.35 ± 1.91 Log IU/mL and 5.56 ± 1.59 Log IU/mL for liver inflammatory activity <A2 and ≥ A2; 6.44 ± 1.87 Log IU/mL and 5.39 ± 1.64 Log IU/mL for liver fibrosis < F2 and ≥ F2; and 6.47±1.88 Log IU/mL and 5.52 ± 1.67 Log IU/mL at treatment indication (< A2 and < F2) and (≥ A2 or/and ≥ F2), respectively. In the propensity score-matched pairs, as treatment indication (< A2 and < F2) and (≥ A2 or/and ≥ F2), the mean HBV DNA levels were 7.90 ± 0.49 Log IU/mL and 5.81 ± 1.23 Log IU/mL (EASL guidelines), 8.01 ± 0.43 Log IU/mL and 5.78 ± 1.42 Log IU/mL (CMA guidelines), respectively.

Obviously, it is unreasonable to set a defined value of HBV DNA to judge the state of CHB disease (natural course) and to guide whether to start treatment. First, despite the correlation between the HBV DNA level and the severity of the disease, the results are not consistent[10-13,37-39]. In this study, regardless of ALT values, patients with HBV DNA low/moderate replication had more serious liver disease. The high level of HBV DNA replication causes the deficiency and dysfunction of the HBsAg specific cytotoxic T lymphocytes, leading to the consequent immune tolerance. However, during the prolonged reproduction, HBV interacts with the host immune system, which can induce a cumulative

Table 4 Predictors of significant liver histology in propensity score-matched pairs

Variables	EASL guidelines (316 pairs)				CMA guidelines (277 pairs)			
	≥ A2 or/and ≥ F2	< A2 and < F2	χ^2/t/Z	SMD	≥ A2 or/and ≥ F2	< A2 and < F2	χ^2/t/Z	SMD
Male, *n* (%)	171 (54.11)	168 (53.16)	0.057	0.811	138 (49.82)	153 (55.23)	1.629	0.202
HBeAg positive, *n* (%)	305 (96.52)	306 (96.84)	0.049	0.824	271 (97.83)	271 (97.83)	0	1.000
Age, mean ± SD, yr	32.92 ± 10.13	33.06 ± 9.96	-0.178	0.859	32.54 ± 9.04	33.31 ± 10.07	-0.937	0.349
ALB, mean ± SD, g/L	42.30 ± 4.59	42.65 ± 3.78	-1.046	0.296	42.35 ± 4.81	42.79 ± 3.76	-1.187	0.236
GLB, mean ± SD, g/L	27.74 ± 4.83	27.74 ± 4.26	0.019	0.985	27.23 ± 4.56	27.53 ± 4.25	-0.809	0.419
AGR, mean ± SD	1.57 ± 0.33	1.57 ± 0.28	-0.037	0.971	1.59 ± 0.29	1.59 ± 0.28	0.198	0.843
ALT, mean ± SD, U/L	23.81 ± 8.29	22.78 ± 8.29	1.569	0.117	23.18 ± 9.10	23.00 ± 8.38	0.235	0.814
AST, mean ± SD, U/L	22.13 ± 8.55	22.57 ± 5.76	-0.769	0.442	21.87 ± 7.74	22.72 ± 5.70	-1.465	0.143
ALP, mean ± SD, U/L	71.64 ± 27.61	70.56 ± 27.09	0.495	0.621	72.15 ± 25.92	71.37 ± 27.39	0.345	0.730
GGT, median (Q1-Q3), U/L	16.00 (13.00–23.00)	16.00 (13.00–23.00)	0.780	0.435	16.00 (13.00-24.00)	17.00 (13.00-23.00)	0.590	0.555
WBC count, mean ± SD, $\times 10^9$/L	5.44 ± 1.27	5.47 ± 1.38	-0.276	0.783	5.51 ± 1.31	5.48 ± 1.30	0.274	0.784
NLR, mean ± SD	1.91 ± 0.80	2.01 ± 1.04	-1.431	0.153	1.89 ± 1.01	2.00 ± 1.04	-1.334	0.183
PLT count, mean ± SD, $\times 10^9$/L	183.43 ± 42.01	187.96 ± 47.38	-1.272	0.204	184.38 ± 49.39	187.11 ± 48.08	-0.659	0.510
HBV DNA, mean ± SD, log IU/mL	5.81 ± 1.23	7.90 ± 0.49	-27.967	< 0.001	5.78 ± 1.42	8.01 ± 0.43	-24.922	< 0.001
APRI, median (Q1– Q3)	0.31 (0.23-0.38)	0.29 (0.23-0.38)	0.507	0.612	0.32 (0.22–0.39)	0.30 (0.23-0.38)	-0.147	0.883
FIB-4, median (Q1–Q3)	0.80 (0.49-1.11)	0.80 (0.58-1.12)	1.460	0.144	0.74 (0.50-1.17)	0.82 (0.59-1.13)	-1.110	0.267
LIF-5, mean ± SD	0.37 ± 0.14	0.36 ± 0.14	0.903	0.367	0.35 ± 0.14	0.36± 0.14	-0.187	0.852

Low/moderate replication, hepatitis B virus (HBV) DNA ≤ 10^7 IU/mL; high replication, HBV DNA > 10^7 IU/mL. Quantitative data of normal distribution were expressed as mean ± standard deviation, non-normal distribution data were expressed as median (Q1-Q3), and categorical data were expressed as frequency and percentage. AGR: Albumin–globulin ratio; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate transaminase to platelet ratio index, APRI = [(AST/ULN)/platelet counts (10^9/L)] × 100[16]; AST: Aspartate aminotransferase; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver; F: Liver fibrosis; GLB: Globulin; FIB-4: Fibrosis-4, FIB-4 = [age (year) × AST (U/L)]/(platelet count (10^9/L) × [ALT (U/L)$^{1/2}$][17]; GGT: Gamma-glutamyl transpeptidase; HBeAg: Hepatitis B e antigen; LIF-5: Liver inflammation and fibrosis-5, LIF-5 = 0.725 + 0.005 × age (year) + 0.003 × ALT (U/L) + 0.004 × AST (U/L) - 0.201 × (A/G) - 0.002 × PLT (10^9/L)[18]; NLR: Neutrophils lymphocytes ratio; PLT: Platelets; WBC: White blood cell; HBV: Hepatitis B virus.

immune damage. The hepatocytes suffer occult and persistent pathological apoptosis, with HBV DNA decreases accordingly, while the liver damage continues[40].

Therefore, in the absence of liver pathology, can individuals with HBV DNA < 2 × 10^3 IU/mL be identified as the 'inactive carriers' when ALT or/and transient elastography (TE) and other indicators are normal? However, whether individuals with 2 × 10^3 ≤ HBV DNA ≤ 10^7 IU/mL can be identified as immune tolerant is still unclear (the indeterminate phase). Second, the correlation ship between the HBV DNA level and the progression of end-stage liver disease (such as HCC) is still controversial. Patients in the indeterminate phase without antiviral therapy had significantly higher risk of developing HCC than those in inactive phase[20,41]. Although patients with high HBV DNA level have a heighten risk of HCC progression in the immune tolerant phase, different studies have still held different views [42-44]. This study has shown the same option with the other studies that the proportion of patients with significant liver tissue damage and HCC progression in the immune tolerant phase is relatively low[43,44], and whether conduct antiviral therapy for them has always been a hot controversy topic on

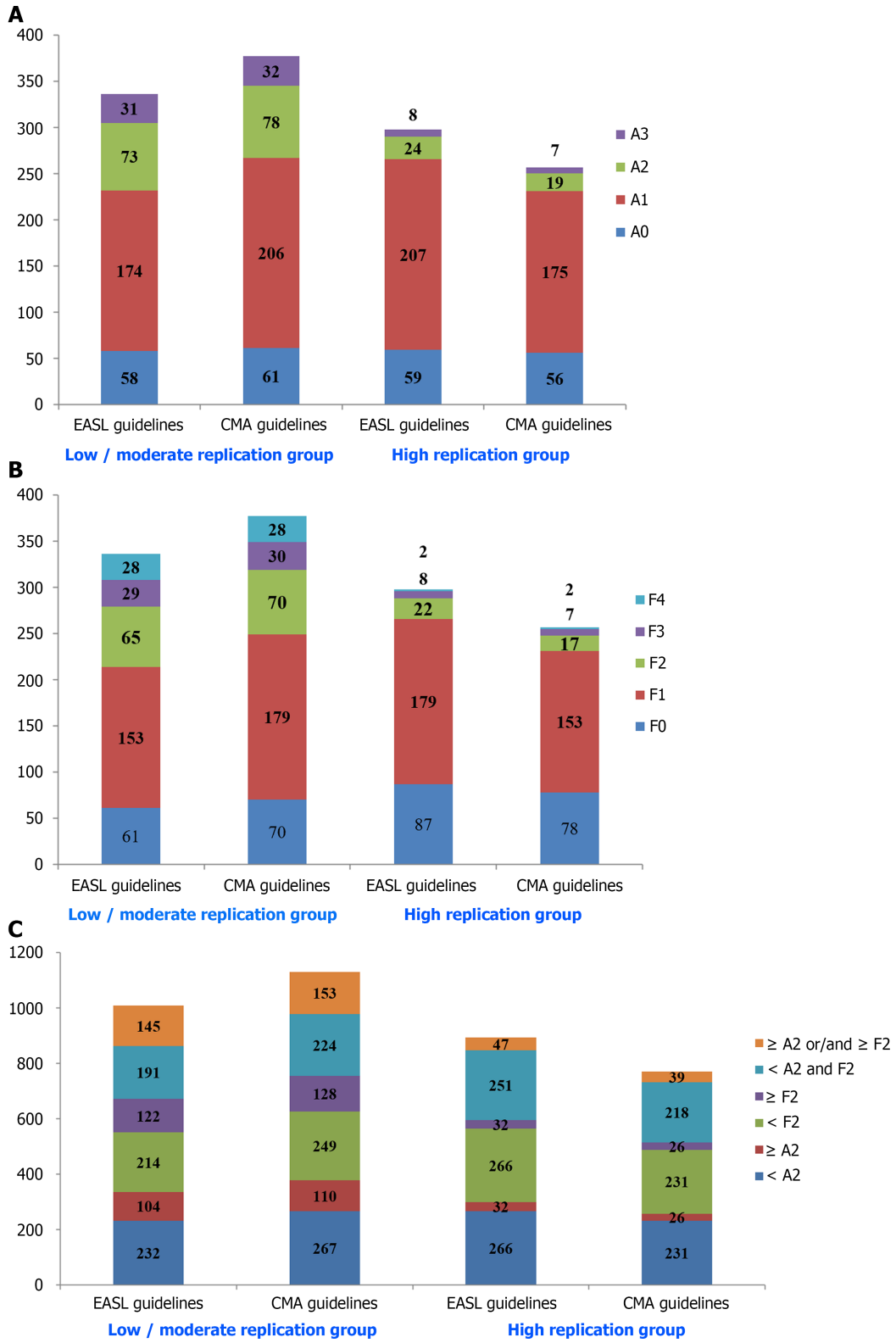


Figure 2 Liver pathological changes among 634 patients with normal alanine transaminase at different hepatitis B virus DNA levels. A: Necroinflammatory activity grading among patients at different hepatitis B virus (HBV) DNA levels; B: Liver fibrosis staging among patients at different HBV DNA levels; C: Liver histopathological severity ≥ A2 or/and ≥ F2 among patients at different HBV DNA levels. A: Liver inflammatory activity; F: Liver fibrosis; CMA: Chinese Medical Association; EASL: European Association for the Study of the Liver.

clinic. The patients with a low HBV DNA level (low-level viremia) still have high risk of disease progression[13,45-48]. Third, for CHB patients in the immune active phase, the defined value of HBV DNA exceeds 2×10^3 IU/mL when HBeAg is negative, while the value of HBV DNA exceeds 10^7 IU/mL or 2×10^7 IU/mL when HBeAg is positive, for which needs further exploration.

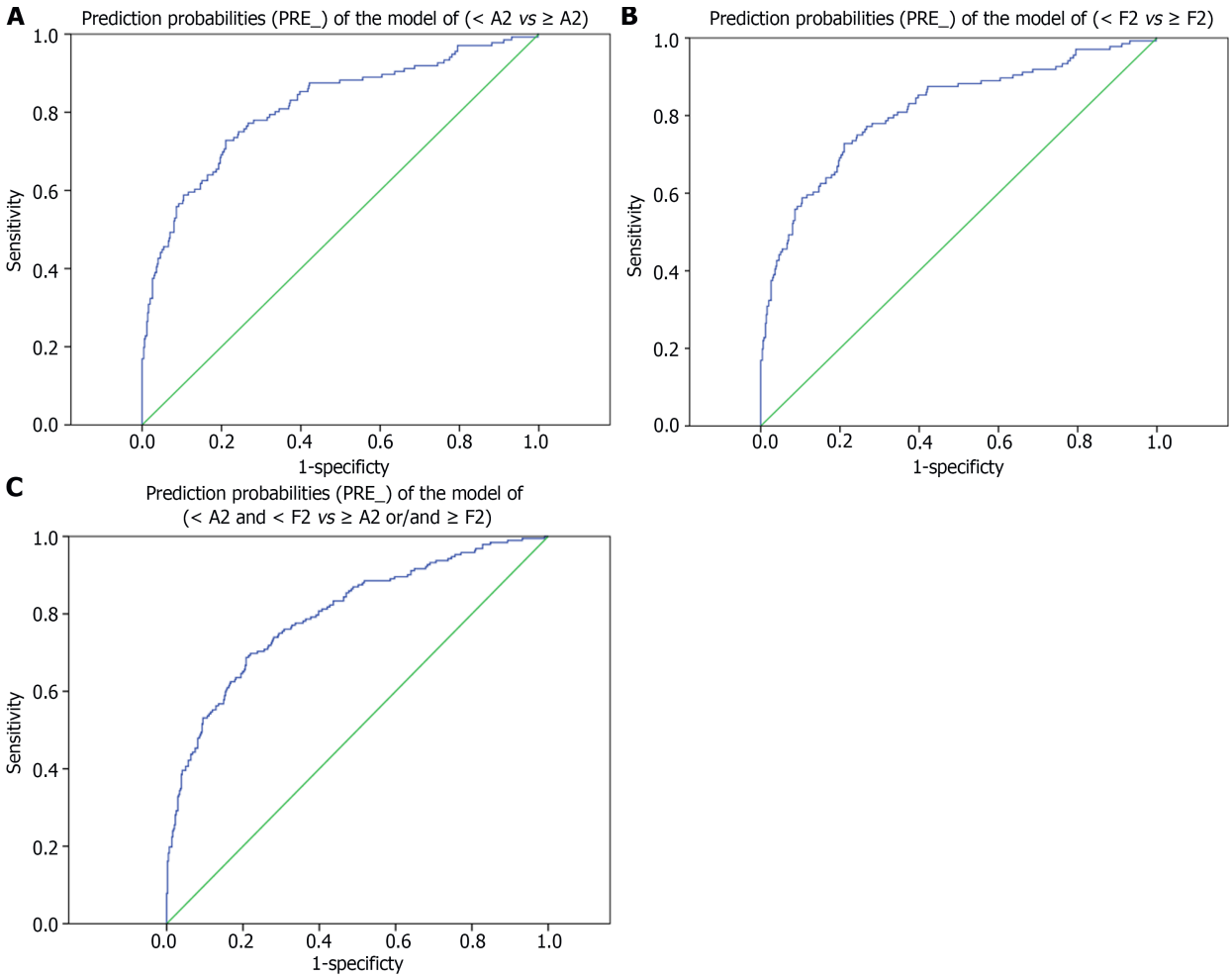


Figure 3 Prediction probabilities (PRE_) of the binary logistic regression model. A: < A2 *vs* ≥ A2; B: < F2 *vs* ≥ F2; C: < A2 and < F2 *vs* ≥ A2 or/and ≥ F2). A: Liver inflammatory activity; F: Liver fibrosis.

There were some limitations in this study. First, the ULN of ALT was 40 U/L, while the AASLD guidelines recommend 35 IU/L for men and 25 IU/L for women[6]. The lower ULN of ALT may help us find more suitable patients needed for treatment. Second, this study had a large time span, the patients enrolled earlier had no TE results due to the absence of the Fibroscan test. Third, it was a cross-sectional study, thus lacking follow-up data. The last, this study didn't determine the HBV genotypes. The dominant genotypes in China are genotype B and C with higher incidence of mother to child transmission, and genotype C infections are more prone to progress to HCC earlier[2,40]. These limitations need to be addressed in the further studies.

CONCLUSION

In summary, this study analyzed the risk factors for liver histopathological severity in CHB patients with normal ALT. It found that HBV DNA (negative correlation) was an independent risk factor for liver disease progression. Because of the widespread use of first-line antiviral drugs and the underlying idea of 'no virus, no disease', it was presumed that the states of CHB disease (natural course) were not that suitable judged by the defined values of HBV DNA level. The classification of CHB may be revised based on whether HBV DNA exceeds the detection value. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) should receive antiviral therapy.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B (CHB) patients can be divided into treatment indication and non-treatment indication individuals. Normal alanine transaminase (ALT) patients in 'immune-tolerant' phase with hepatitis B virus (HBV) DNA higher than 10^7 or 2×10^7 IU/mL and in 'inactive-carrier' phase with HBV DNA lower than 2×10^3 IU/mL do not require antiviral therapy. In fact, we should pay more attention to those who do not match the treatment indications (gray-zone patients both in the indeterminate phase and in the 'inactive-carrier' phase).

Research motivation

In order to analyze the correlation of HBV DNA level and liver histopathological severity, and to explore the significance of HBV DNA for CHB with normal ALT. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) may have severe liver disease pathologically and hematologically.

Research objectives

The states of CHB disease (natural course) were not that suitable judged by the defined values of HBV DNA level. The classification of CHB may be revised based on whether HBV DNA exceeds the detection value. Patients who are in the indeterminate phase or regarded as the 'inactive carriers' (low HBV DNA, low-level viremia) should receive antiviral therapy.

Research methods

From January 2017 to December 2021, a retrospective cross-sectional set of 1299 patients with chronic HBV infection (HBV DNA > 30 IU/mL) who underwent liver biopsy from four hospitals, including 634 with ALT less than 40 U/L. The degrees of liver necroinflammatory activity and liver fibrosis were evaluated according to the Metavir system. Patients were divided into two groups: Low/moderate replication group, HBV DNA ≤ 10^7 IU/mL [the European Association for the Study of the Liver (EASL) guidelines] or ≤ 2×10^7 IU/mL [the Chinese Medical Association (CMA) guidelines]; high replication group, HBV DNA > 10^7 IU/mL or > 2×10^7 IU/mL. Relevant factors for liver histopathological severity were analyzed by univariate analysis, logistics analysis and propensity score-matched analysis.

Research results

At entry, there were 21.45%, 24.29%, and 30.28% of the patients had liver histopathological severities with ≥ A2, ≥ F2, and ≥ A2 or/and ≥ F2, respectively. HBV DNA level (negative correlation) and noninvasive model liver fibrosis 5 value (positive correlation) were independent risk factors for liver histopathological severities (liver necroinflammation, liver fibrosis, and treatment indication). HBV DNA level (negative correlation) was still an independent risk factor when diagnostic models were excluded. For the propensity score-matched pairs, whether based on EASL guidelines or CMA guidelines, the group with significant liver histology damage (≥ A2 or/and ≥ F2) showed much lower HBV DNA level than the group with non- significant liver histology damage (< A2 and < F2). Patients in the moderate replication group (with indeterminate phase) had the most serious liver disease pathologically and hematologically, followed by patients in the low replication group (with 'inactive-carrier' phase) and then the high replication group (with 'immune-tolerant' phase).

Research conclusions

HBV DNA level is a negative risk factor for liver damage. The phase definition of CHB may be revised by whether the level of HBV DNA exceeds the detection low limit value. Normal ALT patients who are in the indeterminate phase or 'inactive carriers' should receive antiviral therapy.

Research perspectives

How to define the natural history of chronic HBV infection and how to identify the patients with normal ALT who need treatment?

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones

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Abstract

BACKGROUND

To date, endoscopic retrograde cholangiopancreatography has become a well-established treatment for common bile duct (CBD) stones. However, it is not suitable for some special patients, such as pregnant women, children or those who cannot stop taking anti-coagulation/anti-platelet agents because of radiation injury and the risk of postoperative bleeding resulting from endoscopic sphincterotomy. To overcome these two problems, this study introduced cholangioscopy-assisted extraction through a novel papillary support for small-calibre and sediment-like CBD stones.

AIM

To assess the feasibility and safety of cholangioscopy-assisted extraction through a novel papillary support (CEPTS) for small-calibre and sediment-like common bile duct (CBD) stones.

METHODS

This Retrospective study was approved by the Ethics Committee of the Chinese PLA General Hospital. We designed a covered single dumbbell-style papillary support between 2021 and 2022. Between July 2022 and September 2022, 7 consecutive patients with small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones underwent CETPS procedures in our center. The clinical characteristics and treatment outcomes of these 7 patients were extracted from a prospectively collected database. And the related data were analyzed. Informed consent was obtained from all participating patients.

RESULTS

A total of 2 patients had yellow sediment-like CBD stones, and aspiration extraction was performed after the insertion of papillary support. Of the 5 patients with clumpy CBD stones (0.4-1.0 cm), 2 underwent basket extraction under direct

vision for a single stone (0.5-1.0 cm, black and black grey), 1 underwent balloon plus aspiration extraction under direct vision for 5 stones (0.4-0.6 cm, brown), and 2 underwent aspiration extraction only for a single stone (0.5-0.6 cm, yellow, none). Technical success, namely, no residual stones in the CBD or left and right hepatic ducts, was achieved in all 7 cases (100%). The median operating time was 45.0 minutes (range 13.0–87.0 minutes). Postoperative pancreatitis (PEP) occurred in one case (14.3%). Hyperamylasaemia without abdominal pain was noted in 2 of 7 patients. No residual stones or cholangitis were found during the follow-up.

CONCLUSION

CETPS appeared to be feasible to treat patients with small-calibre or sediment-like CBD stones. Patients, especially pregnant women and those who cannot stop anticoagulation/anti-platelet agents, could benefit from this technique.

Key Words: Cholangioscopy; Common bile duct stones; Papillary support

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Core Tip: Endoscopic retrograde cholangiopancreatography (ERCP) has become a well-established treatment for common bile duct (CBD) stones. However, the standard ERCP technique always requires endoscopic sphincterotomy (EST), which might lead to the loss of sphincter of Oddi function and some adverse events, including bleeding and perforation. Moreover, radiation injury makes the standard ERCP technique unsuitable for special patients, such as pregnant women and children. To overcome these problems, the present study introduced cholangioscopy-assisted extraction through a novel papillary support for small-calibre and sediment-like CBD stones, reducing radiation injury and avoiding EST.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) has become the standard treatment for common bile duct (CBD) stones[1,2]. However, how to reduce radiation injury for special patients, including pregnant women and children, during this technique remains an important issue. Moreover, endoscopic sphincterotomy (EST), which is always necessary during stone extraction procedures, might lead to the loss of sphincter of Oddi (SO) function, some early adverse events, including bleeding and perforation, and some late adverse events, such as cholangitis, malignant degeneration and recurrent CBD stones, owing to free duodenobiliary reflux[3-5]. Importantly, EST was not appropriate for those patients who could not stop taking anti-coagulation/anti-platelet agents.

The emergence of peroral cholangioscopy made it possible to remove CBD stones under direct vison with less radiation injury[6,7]. However, the relatively difficult operation hindered the further development of this technique. In terms of EST, some studies introduce the self-expandable metal stent (SEMS) to avoid EST[8,9]. However, the SEMS was relatively too long and not desirable.

To overcome the problems mentioned above, we optimized the existing metal stent and made a kind of single dumbbell-style papillary support to facilitate cholangioscopy-assisted stone extraction, avoid EST and preserve SO function. In this study, we introduced cholangioscopy-assisted extraction through a novel papillary support (CETPS) for small-calibre and sediment-like CBD stones. This technique combined the advantages of the preservation of SO function and cholangioscopy-assisted stone extraction under direct vision with less radiation injury.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Ethics Committee of the Chinese PLA General Hospital. Informed consent was obtained from all participating patients. Of note, some instruments, including the cholangioscope, basket and papillary support, were free for patients in this study.

Endoscopists made the clinical decision to perform CETPS for patients diagnosed with small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones by magnetic resonance cholangiopancreatography (MRCP) and/or endoscopic ultrasound (EUS). Patients undergoing CETPS between July 2022 and September 2022 were enrolled in this study. Related data were extracted from a prospectively collected database.

The stone size was measured under MRCP on the maximal cross section. The definition of operation time was the course between the endoscope inserting into and withdrawing from the body. Technical success was defined as no residual stones in the CBD and left and right hepatic ducts, which was confirmed under cholangioscopy after stone extraction.

CETPS procedure

All procedures were performed by one endoscopist who has experience performing more than 2000 ERCP procedures in total.

First, biliary intubation was conducted. Second, a covered single dumbbell-style support (12 mm in diameter, 25-30 mm in length) was placed in the distal CBD and papilla (Figure 1A). Third, for sediment-like CBD stones, endoscopic aspiration was performed under negative pressure (Figures 1B and 2A); for single clumpy CBD stones, a cholangioscope (Micro-Tech, eyeMax, 9F) was inserted into the CBD, and basket extraction was performed through the working tunnel of the cholangioscope under direct vision (Figures 1C-E, 2B and Video 1); for multiple clumpy CBD stones, a cholangioscope (Micro-Tech, eyeMax, 11F) was inserted into the CBD, and balloon extraction was performed through the working tunnel of the cholangioscope under direct vision (Figures 1F and G, 2C and Video 1). Fourth, the cholangioscope was inserted into the CBD again to confirm whether there were remnant stones. Finally, the papillary support was removed (Figure 1H and I).

Postoperative management

Patients underwent routine blood examination and amylase and lipase tests 24 and 72 h postoperatively. Patients underwent computed tomography (CT) and/or endoscopy if abnormal symptoms or blood test parameters were found postoperatively.

Patients were fasted for 1 day after the procedure, and a liquid diet was followed for an additional 1 day if no adverse events occurred. The diet was gradually restored to normal starting on the third day. Postoperative medications mainly included a double-dose proton pump inhibitor and antibiotics for one day. Somatostatin was used if PEP occurred, and the fasting time was extended accordingly. Of note, of the 7 patients, 3 had gallstones and underwent cholecystectomy within 1 mo after CETPS to avoid the recurrence of CBD stones.

Follow-up with a routine duodenoscope was performed at 2 wk postoperatively to observe the papillary morphology.

Adverse events

In the article, PEP, postoperative bleeding, perforation and cholangitis were regarded as major adverse events. The diagnostic criteria of postoperative bleeding was based on the onset of clinical symptoms, including melena or haematemesis or a decrease > 2 g/dL in haemoglobin level. The present study used the diagnostic and classification criteria of PEP proposed by Cotton *et al*[10]: (1) New or worsened abdominal pain; (2) Serum amylase at least three times the upper limit of normal, measured more than 24 h after the procedure; and (3) New or prolonged hospitalization for at least 2 days.

Statistical analysis

Nonparametric data are expressed as medians.

RESULTS

Between July 2022 and September 2022, 7 patients underwent CETPS at the Chinese PLA General Hospital. Clinical characteristics and treatment outcomes for the 7 patients are shown in Table 1.

A total of 2 patients had yellow sediment-like CBD stones, and aspiration extraction was performed after the insertion of papillary support. Of the 5 patients with clumpy CBD stones (0.4-1.0 cm), 2 underwent basket extraction under direct vision for a single stone (0.5-1.0 cm, black and black grey), 1 underwent balloon plus aspiration extraction under direct vision for 5 stones (0.4-0.6 cm, brown), and 2 underwent aspiration extraction only for a single stone (0.5-0.6 cm, yellow, none).

Technical success, namely, no residual stones in the CBD or left and right hepatic ducts, was achieved in all 7 cases (100%). The median operating time was 45.0 min (range 13.0–87.0 min).

Mild PEP occurred in one case (14.3%). Hyperamylasaemia without abdominal pain was noted in 2 of 7 patients. No residual stones or cholangitis were found during the follow-up.

In one case, the papillary support was dilated by an extraction balloon before the insertion of the cholangioscope, given that the papillary was not dilated enough after the insertion of support.

Table 1 Clinical characteristics and treatment outcomes for 7 patients with choledocholithiasis undergoing cholangioscopy-assisted extraction through novel papillary support

Case No.	Age (yr)/sex	Chief complaint	Stone size (cm)[1]	Stone colour	Number of stones	Operation time (min)	Balloon dilatation	Extraction method	Postoperative adverse events
1	44/female	Abdominal pain	0.6	None	1	64	Yes	Aspiration	Postoperative pancreatitis
2	54/female	Abdominal pain	1.0	Black grey	1	53	No	Basket	Hyperamylasaemia
3	25/female	Abdominal pain	0.4-0.6	Brown	5	45	No	Balloon and aspiration	None
4	68/male	Abdominal pain	0.5	Black	1	87	No	Basket	None
5	29/female	Abdominal pain	Sediment-like	Yellow	Sediment-like	13	No	Aspiration	None
6	83/male	Abdominal pain	Sediment-like	Yellow	Sediment-like	31	No	Aspiration	Hyperamylasaemia
7	32/female	Abdominal pain	0.5 cm	Yellow	1	39	No	Aspiration	None

[1]The stone size was measured under computerized tomography on the maximal cross section.

DISCUSSION

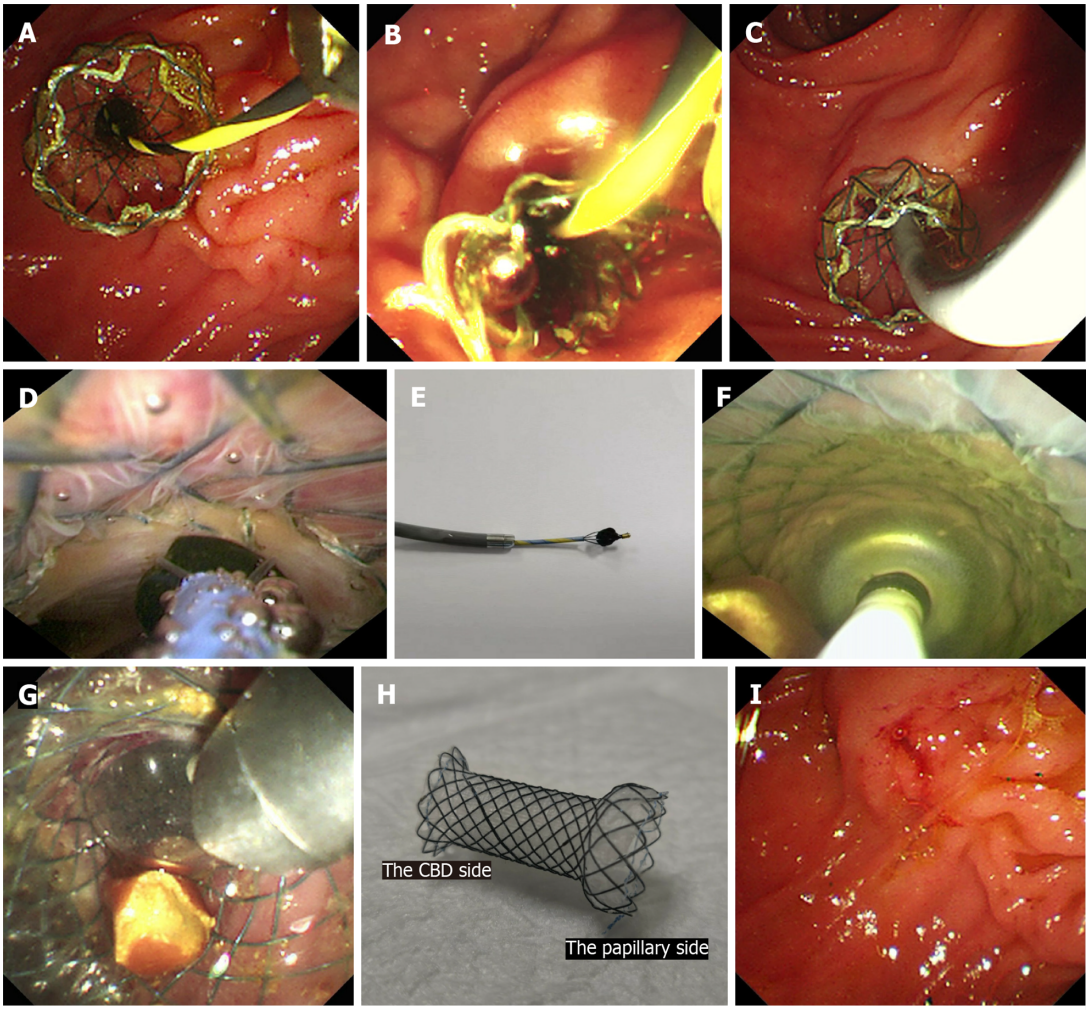
This study showed that CETPS appears to be a feasible and safe treatment option for small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones with a 100.0% (7/7) technical success rate and a 14.3% (1/7) PEP rate without other adverse events, such perforation, bleeding and cholangitis.

The major advantage of CETPS over ERCP with EST for CBD stones is that SO function is retained, avoiding relevant adverse events, including bleeding, perforation, malignant degeneration and recurrent CBD stones[11-13]. Jun *et al*[8] has confirmed that the use of transpapillary SEMSs was effective in the preservation of SO function. Moreover, Cho *et al*[9] concluded that SEMSs can be used for the extraction of CBD stones in patients on dual antiplatelet agents and do not lead to haemorrhagic or thromboembolic events. Of note, the papillary support introduced in the present study has the following merits compared with the SEMSs used in previous studies[8,9]: (1) The novel papillary support was shorter (25-30 mm) than other SEMSs (40-50 mm); (2) the CBD side of the support had a 70-degree angle; and (3) the papillary side of the support had a single dumbbell-style design. In the case of distal stones, the stones might get stuck in the middle between the relatively long SEMS and CBD wall after stent insertion, so Jun *et al*[8] recommended that the patient's position should be tilted to move the stones from the distal CBD to mid-CBD before stent insertion, although it did not always work. Therefore, we designed a support with a shorter length (25-30 mm) and 70-degree angle on the CBD side (Figure 1H), which could lift the stones into the proximal CBD during the process of stent insertion. On the other hand, the single dumbbell-style design (Figure 1H) on the papillary side could protect the support from entering the CBD due to the persistent friction from the cholangioscope. In terms of the PEP, this study presented a reasonable 14.3% (1/7) rate. Theoretically, the use of support could avoid the unintentional PD insertion of the instructions for stone extraction, which might reduce the PEP rate; however, excessive squeezing from the support would induce PEP. Our next method to improve the support is finding the optimal balance between a sufficient support force and a reasonable PEP rate.

Another important advantage of CETPS over traditional ERCP is how the treatment operation is under direct vision. First, radiation injury for patients and surgeons could be minimized, and special patients, including pregnant women and children, could benefit from this technique. Second, the basket and balloon, designed for cholangioscopy, could be opened in the most appropriate position and frap/hold the stones in a timely manner under direct vison. Moreover, operators could find related adverse events, including CBD perforation, bleeding and injury, in a timely manner.

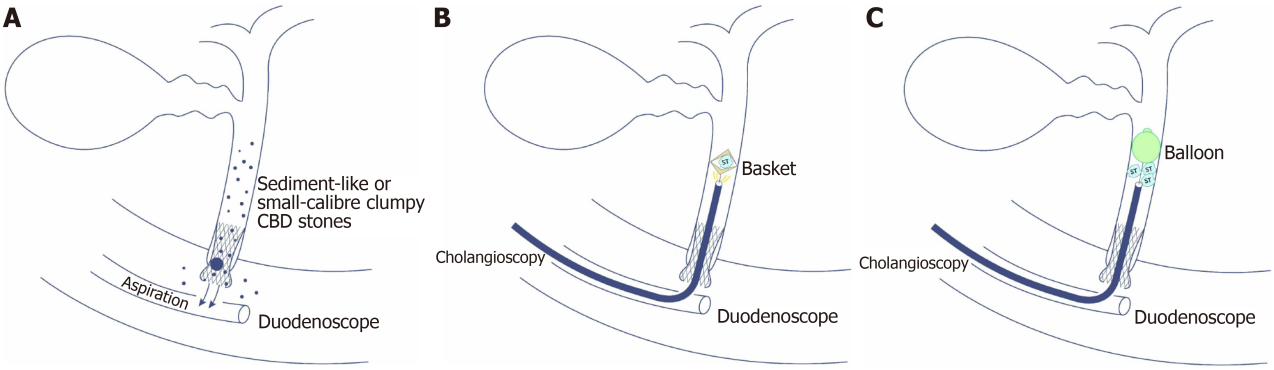
As mentioned above, CETPS combined the advantages of the SEMS technique with the SO function preservation and the cholangioscopy-assisted treatment operation under direct vision. Moreover, the application of novel papillary support established a smooth passageway for the ingress and egress of the cholangioscope and thus facilitated the operation of cholangioscopy-assisted extraction.

The major limitation of our study was the relatively small sample size. However, this paper introduced a novel papillary support that avoids EST during ERCP. Moreover, cholangioscopy-assisted stone extraction using a balloon under direct vision (Table 1, case 3) has not yet been reported.



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Figure 1 The procedures of cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones. A: The novel papillary support was placed in the lower common bile duct (CBD) and papilla; B: Many sediment-like CBD stones flowed from the support under endoscopic aspiration; C: The cholangioscope (Micro-Tech, eyeMax) was inserted into the CBD; D: The single clumpy CBD stone was collected by the basket under cholangioscopy; E: The stone, collected by the basket, was extracted from the CBD and body along with the cholangioscope; F: Multiple clumpy CBD stones were held by the balloon under cholangioscopy; G: One stone, held by the balloon, was extracted from the CBD along with the cholangioscope; H: The novel single dumbbell-style papillary support was removed from the body; I: Papillary morphology immediately after the removal of papillary support.



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Figure 2 The sketch map of cholangioscopy-assisted extraction through novel papillary support for small-calibre and sediment-like common bile duct stones. A: The sketch map of cholangioscopy-assisted aspiration extraction through the support for small-calibre or sediment-like common bile duct (CBD) stones; B: The sketch map of cholangioscopy-assisted basket extraction through the support for small-calibre CBD stones; C: The sketch map of cholangioscopy-assisted balloon extraction through the support for multiple small-calibre CBD stones.

CONCLUSION

In conclusion, CETPS seemed to be a new, feasible and safe treatment strategy for small-calibre (cross diameter ≤ 1.0 cm) or sediment-like CBD stones. Patients, especially pregnant women and those who cannot stop taking anti-coagulation/anti-platelet agents, could benefit from this technique because of the visualized operation and absence of EST. However, further prospective studies with larger populations and longer follow-up periods are warranted.

FOOTNOTES

Author contributions: Zhang WG analyzed the data and wrote the manuscript; Zhang WG and Chai NL contributed equally for this study, and considered as co-first author; Linghu EQ and Chai NL performed the procedures; Zhang B, Li X, Wang JF, Dong H, and Feng YJ revised the manuscript.

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ORIGINAL ARTICLE

Observational Study

Predictive value of presepsin and acylcarnitines for severity and biliary drainage in acute cholangitis

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Abstract

BACKGROUND

Bacteremia, which is a major cause of mortality in patients with acute cholangitis, induces hyperactive immune response and mitochondrial dysfunction. Presepsin is responsible for pathogen recognition by innate immunity. Acylcarnitines are established mitochondrial biomarkers.

AIM

To clarify the early predictive value of presepsin and acylcarnitines as biomarkers of severity of acute cholangitis and the need for biliary drainage.

METHODS

Of 280 patients with acute cholangitis were included and the severity was stratified according to the Tokyo Guidelines 2018. Blood presepsin and plasma acylcarnitines were tested at enrollment by chemiluminescent enzyme immunoassay and ultra-high-performance liquid chromatography-mass spectrometry, respectively.

RESULTS

The concentrations of presepsin, procalcitonin, short- and medium-chain acylcarnitines increased, while long-chain acylcarnitines decreased with the severity of acute cholangitis. The areas under the receiver operating characteristic curves (AUC) of presepsin for diagnosing moderate/severe and severe cholangitis (0.823 and 0.801, respectively) were greater than those of conventional markers. The combination of presepsin, direct bilirubin, alanine aminotransferase, temperature, and butyryl-L-carnitine showed good predictive ability for biliary drainage (AUC: 0.723). Presepsin, procalcitonin, acetyl-L-carnitine, hydroxydodecenoyl-L-carnitine, and temperature were independent predictors of bloodstream infection. After adjusting for severity classification, acetyl-L-carnitine was the only acylcarnitine independently associated with 28-d mortality (hazard ratio 14.396; P <

0.001) (AUC: 0.880). Presepsin concentration showed positive correlation with direct bilirubin or acetyl-L-carnitine.

CONCLUSION

Presepsin could serve as a specific biomarker to predict the severity of acute cholangitis and need for biliary drainage. Acetyl-L-carnitine is a potential prognostic factor for patients with acute cholangitis. Innate immune response was associated with mitochondrial metabolic dysfunction in acute cholangitis.

Key Words: Acute cholangitis; Severity; Biliary drainage; Presepsin; Acylcarnitines

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Core Tip: Acute cholangitis leads to sepsis and organ dysfunction because of biliary obstruction. Identification of predictive biomarkers for patients who require emergent biliary drainage and patients who may progress to systemic bloodstream infection at an early stage of the disease is a key imperative. Our study suggests that presepsin and acetyl-L-carnitine may serve as biomarkers to predict the severity of acute cholangitis and the need for biliary drainage. Innate immune response was associated with mitochondrial metabolic dysfunction.

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INTRODUCTION

Acute cholangitis refers to bacterial infection of the extra-hepatic biliary tract which typically occurs in association with bile duct obstruction caused by choledocholithiasis, malignant stricture, or primary sclerosing cholangitis. Approximately 20% to 71% of patients with acute cholangitis progress to bacteremia or sepsis, which may lead to life-threatening organ failure and death[1,2]. Early biliary drainage to remove biliary obstruction is one of the main emergency treatments for acute cholangitis. Therefore, the identification of predictive biomarkers for patients who require emergent biliary drainage and patients who maybe progress to systemic bloodstream infection at an early stage of the disease is a key imperative.

Bacteremia induces hyperactive immune response and mitochondrial dysfunction which alter metabolism[3]. Presepsin, a soluble leukocyte differentiation antigen 14 (CD14) subtype, is responsible for pathogen recognition by innate immunity[4]. Presepsin is a biomarker of systemic inflammation that can facilitate early diagnosis, risk-stratification, and prognostic assessment of patients with sepsis[5-7]. Carnitine is responsible for mitochondrial transport and β oxidation of fatty acids. L-carnitine and acylcarnitines are established mitochondrial biomarkers[8]. Preclinical and recent clinical studies have demonstrated the association of plasma carnitine or acylcarnitines with organ dysfunction and poor survival in sepsis[9-11]. However, it is unknown whether presepsin or specific acylcarnitine species can reflect severity of acute cholangitis and the timing of biliary drainage. Therefore, this study aimed to evaluate the value of presepsin as well as specific acylcarnitines as predictors of severity, bloodstream infection, biliary drainage and prognosis in patients with acute cholangitis.

MATERIALS AND METHODS

Study design

A prospective observational study was conducted to clarify the predictive value of presepsin and acylcarnitines for severity and biliary drainage in patients with acute cholangitis. The primary outcomes were the abilities of presepsin and acylcarnitines to predict severe acute cholangitis compared with procalcitonin. The secondary outcomes included the value of presepsin and acylcarnitines to predict emergency biliary drainage, positive bloodstream infection, and prognosis of acute cholangitis. This study complied with the principles of the Declaration of Helsinki. The Beijing Friendship Hospital Ethics Committee approved the study protocol (No. 2018-P2-063-01). Patients were enrolled after providing written informed consent.

Study participants

This was a single-center study conducted at the emergency department and emergency intensive care unit of Beijing Friendship Hospital, a National Clinical Research Center of Digestive Diseases. Between May 2019 and July 2021, consecutive adult patients who fulfilled the acute cholangitis criteria based on the Tokyo Guidelines 2018 (TG18) for acute cholangitis were enrolled[12]. The severity was stratified as mild, moderate, and severe according to TG18[12]. The exclusion criteria were as follows: (1) Patients with chronic kidney or liver disease who may have increased presepsin or acylcarnitine levels at baseline[13]; (2) HIV infection; (3) pregnant and lactating women; (4) patients with abdominal trauma or history of abdominal surgery in the past seven days; (5) incomplete data about the main study indices (presepsin, acylcarnitines, or blood culture results); and (6) patients who declined to participate.

Data collection

We recorded demographic data, comorbidities, clinical and laboratory data, severity grading of acute cholangitis and biliary drainage data within 48 h after admission. Disease severity was assessed using the sequential organ failure assessment (SOFA) scores. The criteria for implementing biliary drainage were based on the American Society for Gastrointestinal Endoscopy (ASGE) guidelines for the management of cholangitis[14]. Blood samples of all patients and bile samples of patients who were subjected to endoscopic or percutaneous biliary drainage were cultured for aerobic and anaerobic bacteria. Pathogens in blood samples were identified by blood culture and metagenomic next generation sequencing (mNGS). Data for 28-d mortality were collected during follow-up.

Sample collection and processing

Blood samples (5 mL) were collected immediately after admission and stored at 4 °C. 200 μL of these blood samples were extracted to detect presepsin concentration. The remaining blood samples were centrifuged for 10 min at 4500 g and the plasma sample was then stored at -80 °C within 24 h. For acylcarnitines detection, 50 μL plasma sample was drawn into a 2 mL centrifuge tube and mixed with 140 μL methanol and 10 μL internal standard (NSK-B-1) and further centrifuged for 5 minutes at 12000 g. 100 μL supernatant was transferred into 200 μL inner liner before analyses. For mNGS detection, 3 mL blood sample was drawn from patients and centrifuged at 4000 g for 10 min within 8 h after collection. DNA was extracted from plasma using a TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China, No. DP316) according to the manufacturer's operating manual. The extracted DNA specimens were used for the construction of DNA libraries.

Measurements of presepsin

A chemiluminescent enzyme immunoassay was used to test presepsin concentration by a PATHFAST analyzer (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The detection range was 20 pg/mL to 200000 pg/mL. Information regarding conventional inflammatory biomarkers procalcitonin, C-reactive protein (CRP) and other indicators were obtained from the clinical laboratory data.

Determination of acylcarnitines

Plasma acylcarnitines at enrollment were determined by an ultra-high-performance liquid chromatography-mass system (UHPLC-MS, Supplementary file 1) using a Waters XEVO TQ-S Micro triple quadrupole mass spectrometer (Waters Corp, United States). The length of carbon chains was used to define short-chain (C ≤ 5), medium-chain (C6-10) and long-chain acylcarnitines (C ≥ 12) (Supplementary Table 1).

Next-generation sequencing

mNGS testing of the blood samples was performed and analyzed by BGI-Shenzhen, as previously reported[15]. Briefly, the extracted DNA was fragmented to 300 bp. DNA libraries were constructed by end-repair, adapter ligation and PCR amplification using the PMseqTM high throughput gene detection kit for infectious pathogens (combined probe anchored polymerization sequencing method, BGI-Shenzhen, China, No. RM0438), according to the manufacturer's instructions (Supplementary file 2). Using bioinformatics analysis methods and pathogenic microorganism database, the types of pathogenic microorganisms obtained by sequencing were analyzed, and the detection results of each sample were obtained.

Statistical analyses

Factoring a two-sided $\alpha = 0.05$, $\beta = 0.2$, and assuming 50% of patients with mild acute cholangitis[16], it was determined that 268 patients were required for enrollment, *i.e.*, 134 with mild and moderate acute cholangitis and 134 with severe acute cholangitis. This study enrolled 387 patients to account for patients lost to follow-up and patients with incomplete data collection. Continuous variables with non-normal distribution were presented as median (25^{th} to 75^{th} percentile) and compared by Mann-Whitney *U* test or Kruskal-Wallis test. Comparisons between categorical variables were analyzed by Pearson χ^2 test. Significant biomarkers and clinical variables associated with severity, biliary drainage, bloodstream

infection, and 28-d mortality were identified by multivariate logistic regression models. The area under the receiver operating characteristic (ROC) curves were applied to examine the predictive accuracy of presepsin, acylcarnitines, and procalcitonin for severity and biliary drainage. The optimal cutoff levels determined by ROC curves and Youden index were used to dichotomize presepsin, acylcarnitines, procalcitonin, and other independent predictors. The area under the curve (AUC) comparisons were performed using MedCalc Version 13 software (Mariakerke, Belgium). Kaplan-Meier survival curves were established, and between-group differences in 28-d survival were assessed using the log-rank test. The Cox proportional hazard model was used to calculate the hazard ratio (HR) for 28-d mortality. Spearman rank correlation was performed for the correlation analysis. Two-sided P values < 0.05 were considered indicative of statistical significance. SPSS 25.0 software (SPSS, Chicago, IL, United States) was used for statistical analyses.

RESULTS

Characteristics of the study population

From May 2019 through July 2021, 387 patients with acute cholangitis were admitted to the emergency department or EICU. Data from 107 patients were not analyzed because 29 patients did not meet the inclusion criteria, 38 patients refused consent, 35 patients had incomplete main records, and 5 patients were lost to follow-up (Supplementary Figure 1). The remaining 280 patients were enrolled in this study and assigned to the mild group (n = 65), moderate group (n = 84), and severe group (n = 131) based on the TG18 criteria. The age, proportion of patients with biliary drainage, levels of temperature, white blood cell (WBC) count, total bilirubin, direct bilirubin and SOFA score, and 28-d mortality increased with the severity of acute cholangitis, and the differences among the three groups were significant (P < 0.05 for all) (Table 1).

Performance of presepsin to predict the severity of acute cholangitis compared with procalcitonin, CRP, or acylcarnitine

Compared with the mild group, presepsin, procalcitonin, and CRP levels were significantly higher in patients with moderate and severe acute cholangitis (P < 0.001, Table 2). Plasma short chain and medium chain acylcarnitines (C0, C2–C6, C8) increased, while long chain acylcarnitines (C12-C14, C18, C20, C22) decreased with the severity of acute cholangitis (P < 0.05, Table 2). Multivariate logistic regression showed that increased levels of presepsin, WBC, total bilirubin, temperature, and age, and decreased level of tetradecadienyl-L-carnitine (C14:2), but not procalcitonin and CRP levels, were independent predictors of moderate and severe patients, compared with mild patients (Table 3). The AUC of presepsin for predicting moderate/severe cholangitis was 0.823 (sensitivity 0.75; specificity 0.78; cutoff value 1519 pg/mL), which was higher than that of WBC (0.734; P = 0.0189), total bilirubin (0.677; P = 0.0004), temperature (0.598; P < 0.0001), age (0.635; P = 0.0002), and C14:2 (0.678; P = 0.0012) (Figure 1A). Compared with mild/moderate patients, presepsin, procalcitonin and valeryl-L-carnitine (C5) were independently associated with severe cholangitis (Table 3). The AUC of presepsin (0.801; sensitivity 0.82; specificity 0.66; cutoff value 1680 pg/mL) for severe cholangitis was significantly higher than that of procalcitonin (0.696, P = 0.0028) and C5 (0.664, P = 0.0008) (Figure 1B).

Performance of presepsin and acylcarnitines to predict biliary drainage

One hundred and seventy-six of 280 patients underwent biliary drainage. Compared with patients without biliary drainage, patients with biliary drainage had significantly increased temperature, levels of presepsin, total bilirubin, direct bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (P < 0.05 for all), rather than procalcitonin (P = 0.199) and CRP (P = 0.410) levels. On multivariate logistic regression, high presepsin (OR 2.312, P = 0.004), direct bilirubin (OR 1.902, P = 0.027), ALT (OR 1.878, P = 0.022) and temperature (OR 2.108, P = 0.006), and low plasma butyryl-L-carnitine (C4) (OR 3.326, P = 0.001) were identified as independent predictors of biliary drainage (Table 4). The AUC of a combination of these five predictors was 0.723, which was significantly greater compared with presepsin (0.604, P = 0.0001), direct bilirubin (0.600, P < 0.0001), ALT (0.579, P < 0.0001), temperature (0.599, P = 0.0001), and C4 (0.574, P < 0.0001) alone (Figure 1C). In the model composed of these five factors (Table 4), a cutoff score of 3 was associated with 79.6% sensitivity, 54.8% specificity, 63.8% PPV, and 72.9% NPV for predicting biliary drainage (LR + 1.76 and LR– 0.37) (Figure 1D).

The concentrations of presepsin, acylcarnitines, and procalcitonin in patients with positive and negative blood infection

As a substitute for the severity of acute cholangitis, blood infection was identified by blood culture and blood mNGS. Compared to patients with no blood infection (n = 188), patients with blood infection (n = 92) were more likely to require biliary drainage, and had significantly higher temperature, WBC, and SOFA scores (P < 0.05 for all, Supplementary Table 2). The proportion of male patients in the blood infection group was significantly lower than that in the group without blood infection. The positive

Table 1 Clinical characteristics according to severity grading of acute cholangitis

Variables	All cases (*n* = 280)	Mild (*n* = 65)	Moderate (*n* = 84)	Severe (*n* = 131)	*P* value
Demographic data, age (yr)	74 (66, 84)	69 (63, 81)	79.5 (69, 87)	74 (66, 84)	0.001
Male, *n* (%)	166 (59.3)	34 (52.3)	53 (63.1)	79 (60.3)	0.392
Comorbidities, *n* (%)					
CHD	78 (27.9)	19 (29.2)	22 (26.2)	37(28.2)	0.911
Heart failure	23 (8.2)	3 (4.6)	5 (6.0)	15 (11.5)	0.173
Hypertension	87 (31.1)	16 (24.6)	32 (38.1)	39 (29.8)	0.192
CVD	33 (11.8)	4 (6.2)	10 (11.9)	19 (14.5)	0.233
COPD	10 (3.6)	1 (1.5)	2 (2.4)	7 (5.3)	0.313
Diabetes mellitus	64 (22.9)	11 (16.9)	18 (21.4)	35 (26.7)	0.286
Biliary drainage, *n* (%)					0.015
No	104 (37.1)	34 (52.3)	27 (32.1)	43 (35.0)	
ERCP/PTCD	176 (62.9)	31 (47.7)	57 (67.9)	88 (67.2)	
Infection data					
Temperature (℃)	37.5 (36.7, 38.5)	37.2 (36.5, 38.0)	37.5 (36.6, 38.6)	37.8 (36.8, 38.5)	0.044
WBC count (× 10^9/L)	10.73 (7.37, 14.83)	8.10 (6.47, 10.22)	13.00 (8.61, 16.56)	11.35 (7.89, 15.71)	< 0.001
Liver function					
TBIL (μmol/L)	98.69 (62.63, 142.32)	69.26 (48.02, 103.37)	114.40 (77.04, 164.67)	103.99 (74.30, 139.53)	< 0.001
DBIL (μmol/L)	70.29 (42.68, 98.75)	43.20 (26.53, 75.64)	78.90 (58.09, 110.40)	72.07 (44.85, 96.29)	< 0.001
ALT (U/L)	148.00 (80.00, 287.50)	166.00 (63.50, 386.50)	145.00 (84.62, 300.00)	148.00 (77.00, 240.00)	0.626
AST (U/L)	138.05 (82.50, 286.10)	129.50 (69.60, 346.20)	138.80 (96.10, 299.10)	137.85 (72.03, 266.53)	0.744
SOFA score	2 (1, 4)	1 (1, 2)	1 (1, 2)	4 (3, 5)	< 0.001
28-d mortality, *n* (%)	9 (3.2)	0 (0.0)	0 (0.0)	9 (6.9)	0.005

Data expressed as median (P_{25}, P_{75}) or *n* (%). CHD: Coronary heart disease; CVD: Cerebral vascular disease; COPD: Chronic obstructive pulmonary disease; ERCP: Endoscopic retrograde cholangiopancreatography; PTCD: Percutaneous transhepatic biliary drainage; WBC: White blood cell; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SOFA: Sequential organ failure assessment.

rates of blood culture, blood mNGS, and bile culture were 29.3% (82/280), 66.7% (14/21), and 76.7% (135/176), respectively. The most common bacteria identified were *Escherichia coli*, *Klebsiella pneumoniaeleisure* and *Enterococcus faecium*. Blood infection positivity was associated with significantly higher level of presepsin ($P = 0.001$), procalcitonin ($P < 0.001$), acetyl-L-carnitine (C2, $P = 0.009$), propionyl-L-carnitine (C3, $P = 0.035$), hexanoyl-L-carnitine (C6, $P = 0.036$), and hydroxydodecenoyl-L-carnitine (C12:1 OH, $P = 0.018$).

Ability of presepsin, acylcarnitines, and procalcitonin to identify bloodstream infection in acute cholangitis

We dichotomized presepsin, procalcitonin, C2, C3, C6, and C12:1 OH using the optimal cutoff value. After adjusting for sex, severity grading, and SOFA score, presepsin (OR 3.466, $P = 0.018$), procalcitonin (OR 4.054, $P < 0.001$), C2 (OR 3.716, $P = 0.005$), C12:1 OH (OR 3.611, $P = 0.002$), and temperature (OR 1.671, $P = 0.003$) were found to be independent predictors for bloodstream infection (Table 5). The AUC of presepsin for diagnosing blood infection was 0.610 (sensitivity 0.91; specificity 0.32; cut-off 1147.5 pg/mL), but there was no significant difference between presepsin and procalcitonin (AUC: 0.679), C2 (AUC: 0.599), C12:1 OH (AUC: 0.603), and temperature (AUC: 0.639) in this respect (Supplementary Table 3).

Prognostic value of presepsin, acylcarnitines, and procalcitonin for 28-d mortality

The 28-d mortality rate in this study was 3.2%. The characteristics of survivors and non-survivors are illustrated in Supplementary Table 4. Compared with patients who survived, those who died within 28 d had significantly increased presepsin ($P = 0.004$), C2 ($P = 0.001$), and C12:1 OH ($P = 0.004$) (Figure 2A-C), but not increased procalcitonin ($P = 0.591$) and CRP ($P = 0.141$). The AUC of presepsin (0.839), C2

Table 2 Biomarkers according to severity grading of acute cholangitis

Biomarkers	All cases (*n* = 280)	Mild (*n* = 65)	Moderate (*n* = 84)	Severe (*n* = 131)	*P* value
Presepsin (pg/mL)	1864.00 (1169.75, 2765.75)	1053.00 (576.50, 1505.50)	1634.00 (1103.00, 2325.75)	2536.00 (1812.00, 3730.00)	< 0.001
Procalcitonin (ng/mL)	11.32 (2.10, 40.53)	3.57 (0.64, 10.87)	10.70 (1.48, 31.00)	27.43 (4.76, 54.32)	< 0.001
CRP (mg/L)	83.71 (44.23, 153.29)	48.02 (17.00, 103.48)	80.76 (48.39, 143.91)	107.00 (61.18, 174.12)	< 0.001
Acylcarnitines (μmol/L)					
C0	28.54 (19.60, 38.65)	25.39 (18.72, 32.47)	29.65 (19.96, 38.38)	30.00 (21.19, 40.64)	0.042
C2	9.64 (6.16, 14.01)	8.35 (5.23, 12.29)	9.44 (6.67, 12.53)	10.32 (6.43, 15.30)	0.016
C3	0.38 (0.26, 0.60)	0.31 (0.21,0.43)	0.35 (0.26, 0.53)	0.46 (0.29, 0.71)	< 0.001
C4	0.09 (0.06, 0.16)	0.08 (0.06, 0.12)	0.09 (0.05, 0.13)	0.10 (0.07, 0.19)	0.003
C5	0.11 (0.07, 0.18)	0.08 (0.06, 0.18)	0.09 (0.06, 0.14)	0.14 (0.08, 0.24)	< 0.001
C6	0.11 (0.07,0.15)	0.08 (0.06, 0.14)	0.10 (0.06, 0.13)	0.13 (0.08, 0.20)	< 0.001
C8	0.13 (0.10, 0.22)	0.12 (0.08, 0.19)	0.13 (0.10, 0.20)	0.14 (0.11, 0.23)	0.037
C12	0.09 (0.04, 0.18)	0.14 (0.07, 0.28)	0.09 (0.04, 0.17)	0.08 (0.03, 0.13)	< 0.001
C12:1	0.05 (0.03, 0.08)	0.06 (0.04, 0.10)	0.05 (0.03, 0.09)	0.04 (0.02, 0.07)	0.001
C12DC	0.0005 (0.0004, 0.0008)	0.0006 (0.0005, 0.0009)	0.0005 (0.0004, 0.0009)	0.0004 (0.0003, 0.0007)	0.041
C13	0.03 (0.02, 0.07)	0.04 (0.03, 0.07)	0.03 (0.02, 0.08)	0.02 (0.01, 0.05)	0.008
C14	0.02 (0.01, 0.03)	0.03 (0.02, 0.04)	0.02 (0.01, 0.03)	0.02 (0.01, 0.03)	0.001
C14:1	0.09 (0.04, 0.16)	0.16 (0.07, 0.31)	0.09 (0.05, 0.13)	0.06 (0.03, 0.11)	< 0.001
C14:2	0.07 (0.03, 0.14)	0.12 (0.05, 0.22)	0.06 (0.03, 0.13)	0.05 (0.02, 0.10)	< 0.001
C16	0.10 (0.06, 0.15)	0.12 (0.08, 0.17)	0.10 (0.06, 0.14)	0.09 (0.05, 0.13)	0.003
C16:1	0.06 (0.03, 0.12)	0.10 (0.05, 0.16)	0.05 (0.03, 0.10)	0.05 (0.02, 0.12)	0.003
C16:2	0.02 (0.002, 0.06)	0.04 (0.01, 0.09)	0.01 (0.002, 0.05)	0.02 (0.002, 0.05)	0.006
C18:2	0.13 (0.07, 0.21)	0.16 (0.09, 0.24)	0.12 (0.07, 0.18)	0.11 (0.05, 0.22)	0.014
C18 OH	0.0004 (0.0002, 0.0009)	0.004 (0.003, 0.0013)	0.0006 (0.0002, 0.0010)	0.0004 (0.0002, 0.0007)	0.035
C20	0.0010 (0.0004, 0.0018)	0.0013 (0.0009, 0.0029)	0.0013 (0.0005, 0.0020)	0.0007 (0.0003, 0.0014)	0.001
C20:4	0.0013 (0.0007, 0.0023)	0.0018 (0.0009, 0.0021)	0.0018 (0.0008, 0.0037)	0.0012 (0.0005, 0.0020)	0.024
C22	0.0009 (0.0003, 0.0015)	0.0011 (0.0006, 0.0023)	0.0010 (0.0003, 0.0018)	0.0006 (0.0002, 0.0014)	0.009

Data expressed as median (P_{25}, P_{75}). CRP: C-reactive protein; C0: DL-Carnitine; C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C4: Butyryl-L-carnitine; C5: Valeryl-L-carnitine; C6: Hexenoyl-L-carnitine; C8: Octanoyl-L-carnitine; C12: Dodecanoyl-L-carnitine; C12:1: Dodecenoyl-L-carnitine; C12DC: Dodecanedioyl-L-carnitine; C13: Tridecanoyl-L-carnitine; C14: Tetradecanoyl-L-carnitine; C14:1: Tetradecenoyl-L-carnitine; C14:2: Tetradecadienyl-L-carnitine; C16: Hexadecanoyl-L-carnitine; C16:1: Hexadecenoyl-L-carnitine; C16:2: Hexadecadienyl-L-carnitine; C18:2: Octadecadienyl-L-carnitine; C18OH: Hydroxyoctadecanoyl-L-carnitine; C20: Icosyl-L-carnitine; C20:4: Arachidonoyl-L-carnitine; C22: Behenoyl-L-carnitine.

(0.880), and C12:1 OH (0.822) for 28-d mortality was similar to that of SOFA score (0.848, $P > 0.05$ for all) (Figure 2D and Supplementary Table 5). After adjusting for severity of acute cholangitis by multivariate Cox proportional hazard models, high C2 ($P = 0.004$) was the only independent predictor of 28-d mortality, rather than the levels of presepsin (P=0.732), C12: 1 OH ($P = 0.899$), and SOFA score ($P = 0.133$) (Supplementary Table 6). Based on ROC curves for 28-d mortality and Youden index, a cutoff value of 17.07 μmol/L was used to dichotomize C2. Patients with high C2 Level had significantly higher 28-d mortality compared to those with low C2 Level (HR 14.396; 95%CI: 3.599-57.576; $P < 0.001$; Figure 2E).

Correlation of presepsin with acylcarnitines

Increased level of presepsin ($r = 0.424$, $P < 0.001$), procalcitonin ($r = 0.357$, $P < 0.001$), and C2 ($r = 0.208$, $P < 0.001$) showed a significant association with SOFA score. We confirmed that presepsin, but not procalcitonin, showed a significant positive correlation with total bilirubin ($r = 0.290$, $P < 0.001$), direct bilirubin ($r = 0.304$, $P < 0.001$), and C2 ($r = 0.270$, $P < 0.001$) (Supplementary Table 7).

Table 3 Clinical variables and biomarkers associated with severity of acute cholangitis (multivariate logistic regression models)

	Mild *vs* moderate/severe		Mild/moderate *vs* severe	
Variables	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
Presepsin (pg/mL)	1.001 (1.000-1.002)	< 0.001	1.000 (1.000-1.001)	< 0.001
Procalcitonin (ng/mL)	1.018 (0.995-1.041)	0.134	1.021 (1.008-1.035)	0.001
WBC (× 10^9/L)	1.149 (1.050-1.258)	0.003	0.990 (0.945-1.037)	0.676
CRP (mg/L)	1.001 (0.995-1.008)	0.642	1.003 (0.999-1.008)	0.104
TBIL(μmol/L)	1.008 (1.003-1.013)	0.003	0.999 (0.996-1.002)	0.504
Temperature (℃)	1.540 (1.046-2.267)	0.029	1.118 (0.859-1.456)	0.407
Age (yr)	1.051 (1.018-1.085)	0.002	1.000 (0.977-1.022)	0.973
C14:2 (μmol/L)	0.036 (0.002-0.663)	0.025	-	-
C5 (μmol/L)	-	-	11.490(2.213-59.656)	0.004

WBC: White blood cell; CRP: C-reactive protein; TBIL: Total bilirubin; C14:2: Tetradecadienyl-L-carnitine; C5: Valeryl-L-carnitine; OR: Odds ratio; CI: Confidence interval.

Table 4 A model for predicting biliary drainage in acute cholangitis multivariate logistic regression models

Variables	B	SE	Wald	df	OR	95%CI	*P* value	Score
Presepsin > 1868 (pg/mL)	0.838	0.291	8.308	1	2.312	1.308-4.087	0.004	1
C4 ≤ 0.1803 (μmol/L)	1.202	0.347	12.017	1	3.326	1.686-6.561	0.001	1
DBIL > 51.18 (μmol/L)	0.643	0.290	4.912	1	1.902	1.077-3.358	0.027	1
ALT > 159 (U/L)	0.630	0.275	5.254	1	1.878	1.096-3.219	0.022	1
Temperature ≥ 37.3 (℃)	0.746	0.270	7.602	1	2.108	1.241-3.581	0.006	1

C4: Butyryl-L-carnitine; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

The immunologic profile and mitochondrial function of patients with acute cholangitis are similar to those of septic patients. Thus, in this prospective study, we explored the value of presepsin and carnitine metabolites as biomarkers to predict severity, emergency biliary drainage, and prognosis of patients with acute cholangitis. Our results indicated that the ability of presepsin level to predict moderate/severe and severe cholangitis was superior to that of procalcitonin level. High presepsin, direct bilirubin, ALT, temperature, and low C4 were independent predictors of urgent biliary drainage, and the combination of these five predictors significantly improved the predictive accuracy. As a substitute for severity of acute cholangitis, blood infection was found to be independently associated with the biomarkers of presepsin, procalcitonin, C2, and C12:1 OH. High C2 was identified as the only independent predictor of 28-d mortality. Additionally, the positive correlation between presepsin and C2 reflected the association between innate immune response and mitochondrial fatty acid β-oxidation (FAO) impairment during the progression of acute cholangitis.

CD14 are expressed on the surface of innate immune cells and play a role in the activation of innate immune response after recognition of bacteria[4,17]. Presepsin (soluble CD14) has been confirmed as a marker of host response in sepsis patients. Increased presepsin was demonstrated to be associated with organ dysfunction, positive blood culture and mortality in sepsis[4]. This result was consistent with our finding wherein presepsin level was found to identify severe acute cholangitis and bloodstream infection. Animal model of acute obstructive cholangitis demonstrated infiltration of macrophages and neutrophils into the liver sinusoids and around the bile duct leading to coagulopathy[18]. The study of Guicciardi *et al*[19] revealed that macrophages contributed to the pathogenesis of sclerosing cholangitis. These findings suggested the activation of innate immune response in acute cholangitis. In addition, a recent study showed that the conventional septic biomarker procalcitonin which was produced by C cells of the thyroid gland predicted severe but not moderate/severe acute cholangitis with better accuracy than WBC and CRP[16]. Furthermore, our finding demonstrated the superior ability of presepsin to predict severe or moderate/severe cholangitis compared to procalcitonin and other

Table 5 Variables associated with bloodstream infection multivariate logistic regression models

Variables	B	SE	Wald	df	OR	95%CI	*P* value
Presepsin (High *vs* low[1])	1.243	0.524	5.617	1	3.466	1.240-9.689	0.018
Procalcitonin (High *vs* low[2])	1.400	0.401	12.208	1	4.054	1.849-8.889	< 0.001
C2 (High *vs* low[3])	1.313	0.464	7.998	1	3.716	1.496-9.229	0.005
C3 (High *vs* low[4])	0.319	0.400	0.636	1	1.376	0.628-3.015	0.425
C6 (High *vs* low[5])	-0.450	0.436	1.065	1	0.638	0.271-1.499	0.302
C12:1 OH (High *vs* low[6])	1.284	0.410	9.829	1	3.611	1.618-8.058	0.002
Sex	0.282	0.356	0.628	1	1.326	0.660-2.667	0.428
Temperature (°C)	0.513	0.174	8.714	1	1.671	1.188-2.350	0.003
Severity grading	-0.071	0.285	0.062	1	0.932	0.533-1.628	0.804
SOFA score	-0.062	0.081	0.587	1	0.940	0.802-1.101	0.444

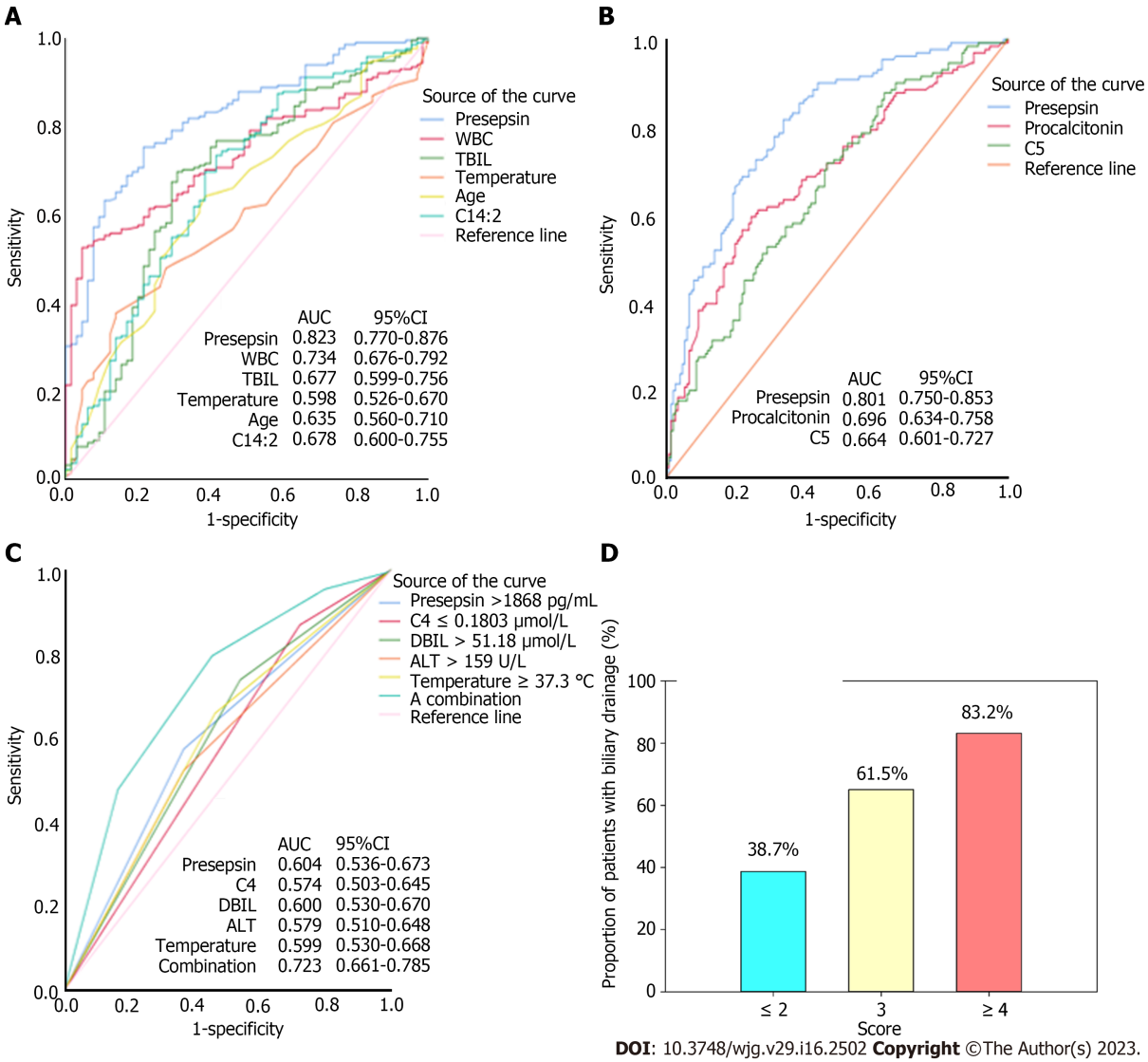
[1]Presepsin (high *vs* low): > 1147.5 pg/mL *vs* ≤ 1147.5 pg/mL.
[2]Procalcitonin (high *vs* low): > 10.83 ng/mL *vs* ≤ 10.83 ng/mL.
[3]C2 (high *vs* low): > 14.59 μmol/L *vs* ≤ 14.59 μmol/L.
[4]C3 (high *vs* low): > 0.53 μmol/L *vs* ≤ 0.53 μmol/L.
[5]C6 (high *vs* low): > 0.12 μmol/L *vs* ≤ 0.12 μmol/L.
[6]C12:1 OH (high *vs* low): > 0.02 μmol/L *vs* ≤ 0.02 μmol/L.
C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C6: Hexanoyl-L-carnitine; C12:1 OH: Hydroxydodecenoyl-L-carnitine; SOFA: Sequential organ failure assessment; OR: Odds ratio; CI: Confidence interval.

markers. The AUC of presepsin was higher than that of other markers in predicting any severity of acute cholangitis. As a surrogate of severe acute cholangitis, predictors for positive bloodstream infection were explored. The most commonly identified bacteria in our study were *Escherichia coli*, *Klebsiella pneumoniaeleisure*, and *Enterococcus faecium,* which is consistent with the findings reported by An *et al*[20]. Similar to the study by Umefune *et al*[16] on the association between procalcitonin and positive blood culture in acute cholangitis, the current study found that presepsin, procalcitonin, C2, and C12:1 OH were independent predictors of positive blood infection.

Additionally, to facilitate early identification of patients who require emergency biliary drainage, we established a predictive model consisting of five factors including presepsin, direct bilirubin, ALT, temperature, and butyryl-L-carnitine (C4). Previous studies suggested that procalcitonin might be a decision-supporting biomarker for urgent biliary decompression even in cases that are not categorized as severe based on TG13[21,22]. However, there was no evidence in this study that procalcitonin, rather than presepsin, could independently predict biliary drainage. The results indicated superior ability of presepsin to reflect the degree of biliary obstruction compared to procalcitonin.

Acylcarnitines are recognized for facilitating FAO for energy production in mitochondria[23]. The blood concentrations of acylcarnitines, which represent a group of mitochondrial-derived metabolites, reflect disorders of long-chain FAO[24]. The production of acetylcarnitine (C2) represents metabolic flexibility in buffering the metabolic status between glucose oxidation and fat oxidation states[25]. Elevation in plasma concentration of C2 is a signal of metabolic inflexibility[8]. Mitochondrial metabolic dysfunction has been implicated as one of the potential causes of organ dysfunction in sepsis[26]. Metabolic flexibility was shown to be an important characteristic of patients with sepsis for survival [27]. Plasma C2 Level was shown to be associated with multiple organ dysfunction, extubation, and freedom from vasopressors, or mortality in patients with sepsis[10,11]. In several studies, plasma short chain and medium chain acylcarnitines (C2, C3, C4, C5, C6, C8, C10) were significantly increased in the non-survivors[11,28-30] and only C2 was associated with all of these indices and 28-d mortality in sepsis [12]. Similar to previous studies, our findings showed that concentrations of short- and medium-chain acylcarnitines increased with the severity and C2 was the only acylcarnitine implicated in 28-d mortality. Increased plasma C2 Level may indicate metabolic inflexibility of nonsurvivors with acute cholangitis. Inconsistent with the absence of long chain acetylcarnitine in sepsis studies, we found that concentrations of long chain acylcarnitines decreased with the severity of acute cholangitis, which might be due to impairment of long-chain FAO with disease progression.

Interestingly, in the current study, reduced butyryl-L-carnitine (C4) was found to be an independent predictor of biliary drainage. Butyrate, a short chain fatty acid, is produced in the bowel by bacterial fermentation of dietary fiber. C4, a butyrate ester of carnitine, is known to help maintain intestinal health and prevent intestinal inflammation[31]. C4 combined with presepsin, direct bilirubin, ALT, and temperature showed better predictive accuracy for emergency biliary drainage. The total score of this model was 5, and 83.2% of patients with score > 4 required biliary drainage (Figure 1D). Moreover, the



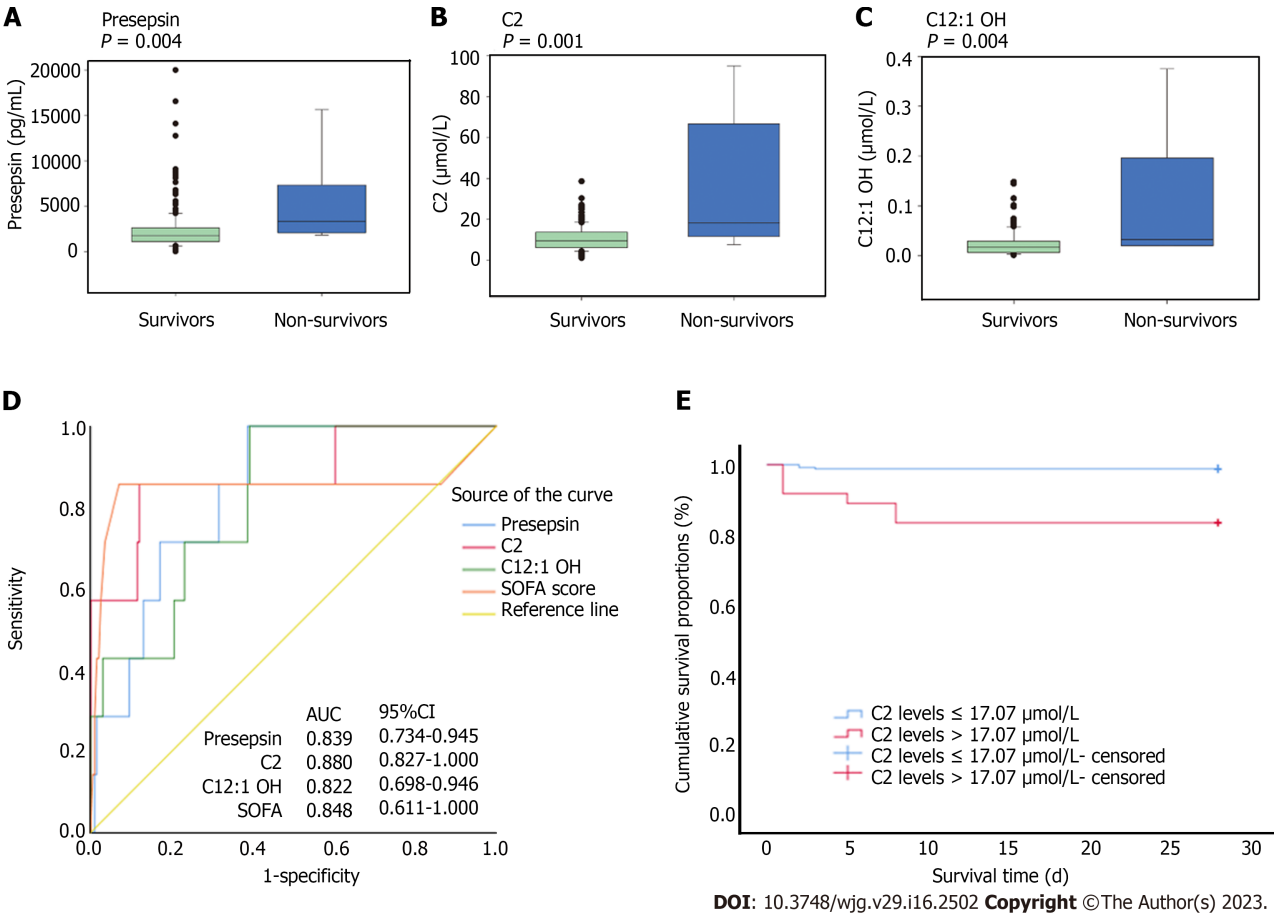
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Figure 1 Receiver operating characteristic curves for biomarkers and clinical parameters for predicting the severity of acute cholangitis and the need for biliary drainage. A: Mild *vs* moderate/severe acute cholangitis; B: Mild/moderate *vs* severe acute cholangitis; C: No biliary drainage *vs* biliary drainage in acute cholangitis; D: Correlation between the score of biliary drainage prediction model and the proportion of patients with biliary drainage. WBC: White blood cell; TBIL: Total bilirubin; C14:2: Tetradecadienyl-L-carnitine; C5: Valeryl-L-carnitine; C4: Butyryl-L-carnitine; ALT: Alanine aminotransferase.

association between C4 and SOFA score, presepsin, and procalcitonin (Supplementary Table 7) may be explained by the compensatory mechanism of intestinal health on intestinal inflammation in acute cholangitis. Furthermore, the association between C2 and inflammation, as well as the hepatic host response to bacteria leading to the accumulation of long-chain acylcarnitines and defective FAO[32], may explain why C2 and hydroxydodecenoyl-L-carnitine (C12:1 OH) were identified as independent predictors of bloodstream infection in acute cholangitis.

The association between innate immunity and FAO may explain the positive correlation between presepsin and acetylcarnitine. Recent evidence suggested that metabolic reprogramming including FAO was a prerequisite for the activation of macrophages and monocytes[33,34]. A study by Zhu *et al*[35] found that the rewiring of metabolic and mitochondrial bioenergetics by monocytes activated, deactivated and resolved acute inflammation in turn. During deactivation, the characteristics of lipid metabolic rewiring included increased acylcarnitines levels. The function of immunocytes depends on specific metabolic programs in mitochondria, including post-translational modifications (*e.g.*, acetylation). In their *in vitro* and *in vivo* studies, Chi *et al*[36] found that histone deacetylase 3 couples mitochondria to deacetylate the FAO enzyme HADHA for NLRP3 inflammasome activation in macrophages.

Some limitations of this study should be considered. First, we did not analyze the dynamic changes in presepsin and acylcarnitines levels over time throughout the disease course. Second, the association of presepsin or acylcarnitines with chronic liver or kidney dysfunction was not assessed in this study. Third, due to the low mortality, a larger sample size was required to verify biomarkers that were associated with death. Fourth, blood mNGS was required for larger population size to improve the



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Figure 2 Association of presepsin and acylcarnitines with 28-d survival in acute cholangitis. A-C: 28-d survival showed significant correlation with presepsin (A), acetyl-L-carnitin (C2; B), and hydroxydodecenoyl-L-carnitine (C12:1 OH; C); D: Receiver operating characteristic curves of presepsin, C2, C12:1 OH and sequential organ failure assessment score for predicting 28-d mortality in patients with acute cholangitis; E: Kaplan–Meier survival curves showed that patients with C2 Levels > 17.07 μmol/L had a lower probability of survival at 28 d (log-rank = 25.01; $P < 0.001$) compared to patients with lower levels in acute cholangitis. C2: Acetyl-L-carnitine; C12:1 OH: Hydroxydodecenoyl-L-carnitine; SOFA: Sequential organ failure assessment; AUC: Area under the curve.

detection rate of positive bloodstream infection.

CONCLUSION

Our study identified presepsin as a specific biomarker to predict the severity and emergency biliary drainage of acute cholangitis compared to procalcitonin and other clinical parameters. Acetyl-L-carnitine might be a promising biomarker for predicting mortality in patients with acute cholangitis. Our findings clarify the association between innate immune responses and mitochondrial FAO impairment in acute cholangitis.

ARTICLE HIGHLIGHTS

Research background

Acute cholangitis is potentially lethal when accompanied by sepsis because of biliary obstruction. It is necessary to identify predictive biomarkers for patients who require emergent biliary drainage and patients who maybe progress to systemic bloodstream infection at an early stage of the disease.

Research motivation

Bacteremia induces hyperactive immune response and mitochondrial dysfunction. Presepsin is responsible for pathogen recognition by innate immunity. Acylcarnitines are established mitochondrial biomarkers. However, it is unknown whether presepsin or specific acylcarnitine species can reflect the severity of acute cholangitis and the timing of biliary drainage.

Research objectives

To clarify the early predictive value of presepsin and acylcarnitines for severity and biliary drainage of acute cholangitis.

Research methods

In this prospective observational study, 280 patients with acute cholangitis were included from May 2019 to July 2021. The severity was stratified as mild, moderate, and severe according to according to the Tokyo Guidelines 2018. Blood presepsin and plasma acylcarnitines were tested at enrollment by chemiluminescent enzyme immunoassay and ultra-high-performance liquid chromatography-mass spec -trometry, respectively. Patients were followed-up for 28 d.

Research results

The concentrations of presepsin, procalcitonin, short- and medium-chain acylcarnitines increased, while long-chain acylcarnitines decreased with the severity of acute cholangitis. The areas under the receiver operating characteristic curves (AUC) of presepsin for diagnosing moderate/severe and severe cholangitis (0.823 and 0.801, respectively) were greater than those of conventional markers. The AUC of a combination of presepsin, direct bilirubin, alanine aminotransferase, temperature, and butyryl-L-carnitine for predicting biliary drainage was 0.723. Presepsin, procalcitonin, acetyl-L-carnitine, hydroxy-dodecenoyl-L-carnitine, and temperature were independent predictors of bloodstream infection. After adjusting for severity classification, acetyl-L-carnitine was the only acylcarnitine independently associated with 28-d mortality (hazard ratio 14.396; $P < 0.001$) (AUC: 0.880). Presepsin concentration showed positive correlation with direct bilirubin and acetyl-L-carnitine.

Research conclusions

Presepsin may serve as a specific biomarker to predict the severity and biliary drainage of acute cholangitis. Acetyl-L-carnitine might be a promising prognostic factor for patients with acute cholangitis. Innate immune response was associated with mitochondrial metabolic dysfunction in acute cholangitis.

Research perspectives

Prospective observational study reports the predictive value of presepsin and acylcarnitines for severity and biliary drainage of acute cholangitis. Future research should focus on the association between acylcarnitines and the changes of intestinal microflora and bacterial translocation in acute cholangitis.

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FOOTNOTES

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